BRITISH MEDICAL BULLETIN
VOLUME 16 NUMBER 1 JANUARY 1960

Scientific Editor: *Lawrence P. Garrod*

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Antibiotics in Medicine: Introduction

Fifteen years ago the only antibiotic available for systemic use was penicillin. The clinician has now to make a choice from about fifteen for the generality of bacterial infections, apart from others having more restricted indications. A few of these, like penicillin, were discovered by independent workers: we owe streptomycin and neomycin to S. A. Waksman, bacitracin to Balbina Johnson, the research associate of the bacteriologically minded surgeon F. L. Meleney, and nystatin, as its name denotes, to members of the staff of the New York State Department of Public Health. But the majority were unearthed—in a literal sense, since world-wide soil surveys have been the means of their discovery—by the enterprise of the pharmaceutical industry, which has also provided the background information justifying ultimate clinical trial, and continues to provide much of the advice on which the practitioner relies in their clinical use. During the same period those engaged in synthetic chemistry have not been idle: several drugs active against tuberculosis have become available, and more names have been added to the already bewildering list of sulphonamides.

The numerous antibiotics now known are not all distinct in their activity: a few are unique in themselves, but the majority fall into groups displaying close similarity of action.

The "spectrum" of penicillin, with high activity against gram-positive organisms, is imitated by the erythromycin group, which includes spiramycin, oleandomycin and possibly streptogramin and staphylomycin, and by bacitracin, novobiocin, vancomycin and ristocetin. Near equality of activity against gram-positive and gram-negative organisms generally is characteristic of the tetracyclines, but their scope embraces rickettsias, the large viruses and to some extent the tubercle bacillus. Chloramphenicol, although in some properties unique, has close affinities with this group. Another somewhat heterogeneous group is characterized by high activity against the tubercle bacillus and against gram-negative organisms, with lesser activity against gram-positive organisms, particularly streptococci. Foremost in this is streptomycin, with which viomycin may be classed solely for its action in tuberculosis: the remainder form the neomycin group, which includes kanamycin, framycetin and paromomycin, the usefulness of which is limited by toxicity. An activity directed exclusively against gram-negative organisms is the peculiar property of polymyxin. Finally there are the polyene antibiotics—nystatin, amphotericin and trichomycin—the only activity of which is against fungi.

Parallel with the discovery of new antibiotics there have been two other developments militating against their successful use. One has been the appearance of untoward effects from their administration, including manifestations of direct toxicity, sensitization phenomena, and the production of superinfections by resistant bacteria which are sometimes more intractable or even more dangerous than the primary disease. The second development, even more threatening to the future usefulness of some of the antibiotics, is the wide-spread acquisition of bacterial resistance to them.

The part played by the pure scientist as distinct from the clinician in this constantly widening field is not to be forgotten. Although bacteriology, pharmacology and chemistry are all concerned, it is the chemist alone who can go to the root of the matter by first ascertaining the structure of an antibiotic and then endeavouring to determine why this chemical configuration confers the power to arrest bacterial growth. If he has not yet fully succeeded in such efforts, a substantial body of knowledge is accumulating on which the rationalization of antibiotic therapy may ultimately be based.

The choice of subjects for an issue of this Bulletin devoted to antibiotics was not an easy matter. To attempt a comprehensive account of therapeutic use was out of the question: the issue could have been filled three times over by accounts of the properties of individual antibiotics, of the treatment of infections by individual organisms, and of the uses of antibiotics in diseases of different systems and in specialized branches of medicine and surgery. Only four contributions are concerned with specific clinical uses, and two of these uses are of a general nature, namely, preventive administration in medicine and surgery. The two individual infections accorded separate consideration are tuberculosis and bacterial endocarditis. Antibiotic treatment has had perhaps a more profound effect on prognosis in these two infections than in any other: in both, the treatment must be patient and prolonged, and the correct choice of antibiotics, based on laboratory studies, is vital to success. The remaining contributions deal with more general matters, from the mode of discovery of antibiotics and their chemical nature and relationships, to the methods of their employment, their behaviour in the body and the ill effects as well as benefits which they can produce. Acquired bacterial resistance is discussed, first from the point of view of how it develops, a subject of acute controversy and of great interest as affording clues to actual modes of action, and secondly in relation to its clinical consequences. Two contributions are on purely technical subjects—the determination of bacterial sensitivity and the employment of antibiotics in selective culture media. It is apt to be forgotten that this was the sole use to which penicillin was put for ten years following its original discovery.

There are aspects of this subject on which it is not yet possible to dogmatize. They include, naturally, those deeper questions involving interaction between the antibiotic and the individual cell of which knowledge is still imperfect. They also include matters of a much more mundane and practical nature, prominent among which is the value of combinations of antibiotics in relation to single ones. The actual necessity for combined treatment is established in tuberculosis and in enterococcal endocarditis: how far does this principle apply in other infections? Is the use of mixtures of antibiotics to be condemned as "shotgun" therapy, or commended as discouraging bacterial resistance and sometimes as exerting a superior therapeutic effect? Another everyday question about which there is much difference of opinion is the extent to which antibiotics can justifiably be used to prevent possible infection, especially in connexion with surgery.

In these and other matters the aim of this issue is to provide the reader with well-ascertained information on which he can base his own opinion. Much of it he will not find elsewhere except in original papers: no development in medicine has ever been at once so rapid and so complex, and would-be authors of books about it may well have been daunted by the fact that anything which takes a long time both to write and to print is likely to be out of date even before publication.

Lawrence P. Garrod

Professor Lawrence P. Garrod acted as Chairman of the planning committee for this symposium and also as Scientific Editor; we are grateful to him for all the help he gave in both capacities.

CHEMISTRY AND CLASSIFICATION OF ANTIBIOTICS

E. P. ABRAHAM M.A. D.Phil. F.R.S.

G. G. F. NEWTON M.C. M.A. D.Phil.

Sir William Dunn School of Pathology
University of Oxford

1 Antibiotics derivable from amino acids or similar units
- *a* D-Cycloserine (oxamycin)
- *b* L-Azaserine and 6-diazo-5-oxo-L-norleucine (DON)
- *c* Chloramphenicol
- *d* Penicillins
- *e* Bacitracins
- *f* Polymyxins
- *g* Actinomycins
- *h* Tyrothricin

2 Antibiotics derivable mainly or partly from acetate
- *a* Griseofulvin
- *b* Tetracyclines
- *c* The erythromycin (macrolide) group
- *d* Polyene antibiotics: nystatin and amphotericin

3 Antibiotics derivable from sugars
- *a* Streptomycin
- *b* Kanamycin
- *c* Neomycin B and C

4 Miscellaneous antibiotics
- *a* Novobiocin
- *b* Vancomycin
- *c* Ristocetin A and B

5 Conclusion
References

The antibiotics that are commonly used in medicine have several biological properties in common. They show a high activity against certain pathogenic micro-organisms, a relatively low toxicity to human tissues, and some resistance to inactivation by enzymes and other constituents of the body. These properties are associated, not surprisingly, with types of chemical structure that have not been encountered in animal biochemistry. The individual structures differ widely among themselves, but they appear to arise from variations on a limited number of biogenetic themes and the latter provide a basis for a rational classification in chemical terms.

Three large collections of antibiotics contain structures or major structural fragments that are derivable mainly from amino acids, acetate and simple sugars respectively. Each of these collections may contain compounds of very dissimilar types, formed by the condensation of similar structural units in different ways. Within them are groups and families of antibiotics, the members of a single family having closely related structures formed, with minor changes, by a common biosynthetic process.

Little is yet known about the primary reactions between antibiotics and microbial cells, and few broad relationships can be discerned between structure and activity. Nevertheless, studies of different members of the same antibiotic family and, in some cases, of similar compounds obtainable by synthesis have provided information about the effect of chemical changes in a single type of molecule on biological properties.

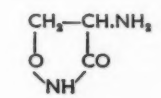
Most of the antibiotics discussed here are used clinically for the treatment of bacterial, rickettsial, or fungal infections, but several compounds that show antitumour activity have also been considered. The substances have been grouped, as far as possible, in a manner designed to illustrate the structural and biogenetic relationships between them.

1. Antibiotics Derivable from Amino Acids or Similar Units

A few antibiotics, such as cycloserine, are structurally related to a single amino acid. Others, such as the penicillins, consist of two or three amino-acid residues condensed to form novel ring systems. Yet others, such as bacitracin, are polypeptides containing from six to 12 amino acids. Certain structural features are characteristic of these substances. For example, some of their amino-acid residues have the D-configuration and some of them may differ structurally from those found in proteins. The chains of the polypeptide antibiotics are curled into large rings closed by peptide or ester linkages, or are terminated by unusual residues.

a. D-*Cycloserine* (*Oxamycin*)

This compound, produced by a *Streptomyces* sp., is D-4-amino-3-isoxazolidone (I) (Kuehl *et al.* 1955). Its structure can be formally derived from that of D-serine amide by



(I)

removal of the elements of water, but whether serine or alanine is an intermediate in its biosynthesis has not been established. D-Cycloserine inhibits the synthesis of the cell wall in *Staphylococcus aureus*. The L-isomer also shows antibacterial activity, but it does not interfere with cell-wall synthesis and must therefore have a different mode of action (Ciak & Hahn, 1959).

b. L-*Azaserine and* 6-*Diazo*-5-*Oxo*-L-*Norleucine* (*DON*)

These two compounds, having structures (II) and (III) respectively, contain a reactive aliphatic diazo group (Dion, Fusari, Jakubowski, Zora & Bartz, 1956).

$$N_2CH.COO.CH_2.CH(NH_2)CO_2H \quad \text{(II)}$$

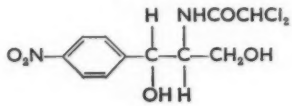
$$N_2CH.CO.CH_2.CH_2.CH(NH_2)CO_2H \quad \text{(III)}$$

Both compounds are produced by *Streptomyces* spp. and both show antitumour activity. Azaserine (*O*-diazoacetyl-L-serine) could be regarded as a derivative of *O*-glycylserine, but Birch (1958) has suggested that it might be formed, by an oxidative process, from ornithine, and that DON might be formed from lysine in a similar manner.

L-Azaserine and DON inhibit the utilization of glutamine for purine biosynthesis, possibly by acting as specific alkylating agents. Both their optical configuration and chain-length are important to their functioning in this way. D-Azaserine and 5-diazo-4-oxo-L-norvaline are inactive (Buchanan, 1958).

c. Chloramphenicol

This widely used antibiotic, produced by *Streptomyces venezuelae*, is D-(−)-*threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1:3-propanediol (IV) and has been obtained synthetically by several routes (Controulis, Rebstock & Crooks, 1949; Long & Troutman, 1949). The presence of a nitro



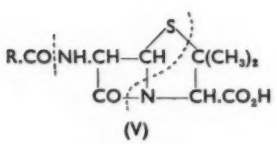
(IV)

group and a dichloroacetyl group is unusual in natural products. The C_6–C_3 skeleton is common and occurs, for example, in the aromatic amino acids phenylalanine and tyrosine, but the biosynthetic pathway leading to the skeleton of chloramphenicol has not yet been elucidated.

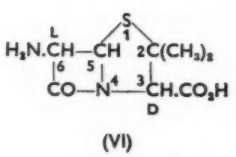
A number of changes can be made in the aromatic ring of chloramphenicol (such as the transfer of the nitro group to the *ortho* or *meta* position, or the replacement of the nitro group by halogen) without greatly reducing antibacterial activity. However, no analogue so far prepared appears to be superior to the natural product as a chemotherapeutic agent. Changes in the side-chain, including alterations of configuration at the two asymmetric centres, result in an almost total loss of activity (Huebner & Scholz, 1951). For example, the (+)-L-*threo* isomer of (IV) has only about 0.5% of the activity of the natural (D-*threo*) chloramphenicol, and the two *erythro* forms are inactive.

d. Penicillins

The penicillins (V) are acyl derivatives of 6-aminopenicillanic acid (VI). Several hundred members of the family have



(V)

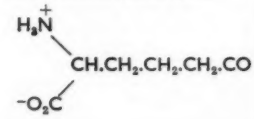


(VI)

been obtained by the addition of suitable acids or their derivatives (RCO_2H) as side-chain precursors to fermentations with *Penicillium chrysogenum* (Behrens, Corse, Edwards, Garrison, Jones, Soper, Van Abeele & Whitehead, 1948). Most of these precursors are monosubstituted acetic acids, the substituent being a non-polar aromatic group or aliphatic chain. A penicillin with a side-chain derived from the highly polar D-α-aminoadipic acid, known as cephalosporin N or synnematin B, is produced by certain species of the genus *Cephalosporium* (Newton & Abraham, 1954). The side-chains of some of the compounds that have been used in medicine are shown in Table I.

Studies with isotopically-labelled amino acids have established that penicillin is formed from cysteine, valine and a side-chain precursor, as shown by the broken lines in (V). L-Cysteinyl-L-valine (or an acyl derivative of it) appears to undergo an oxidative condensation yielding the β-lactam ring. αβ-Dehydrogenation of the L-valine residue, followed by addition of sulphur to the double bond to form a thiazolidine

TABLE I. Nature of the Side-Chains of Certain Penicillins

Side-chain (R.CO)	Penicillin
$CH_3.CH_2.CH{=}CH.CH_2.CO$	Δ^2-Pentenyl (F)
$C_6H_5.CH_2CO$	Benzyl (G)
$C_6H_5.O.CH_2.CO$	Phenoxymethyl (V)
$CH_2{=}CH.CH_2.S.CH_2.CO$	Allylmercaptomethyl (O)
	D-4-Amino-4-carboxy-*n*-butyl (cephalosporin N or synnematin B)

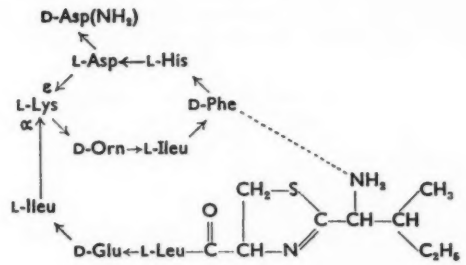
ring with the D-configuration at C_3, could then lead to (VI) or (V) (Arnstein, 1957). 6-Aminopenicillanic acid has been isolated from fermentations with *Penicillium chrysogenum* to which no side-chain precursor was added (Batchelor, Doyle, Nayler & Rolinson, 1959), but it does not necessarily follow that this compound is an intermediate on the main pathway of penicillin biosynthesis.

Penicillin V has been synthesized by ring-closure of the corresponding penicilloate, effected by dicyclohexyl carbodiimide, to form the β-lactam ring (Sheehan & Henery-Logan, 1957). The total synthesis of 6-aminopenicillanic acid (VI) has also been reported (Sheehan, 1958). The properties of synthetic compounds and of the various penicillins obtained by fermentation have revealed some of the ways in which biological properties depend on structural features. It seems that the 6-aminopenicillanic acid nucleus must be intact and that all the asymmetric centres in this nucleus must have the natural configuration for the corresponding penicillin to show high antibacterial activity. For example, a penicillin with the L-configuration at C_3 appears to be inactive. The nucleus itself shows only a low activity. Acylation of the nucleus with a variety of non-polar groups, such as the first four shown in Table I, yields penicillins with a high activity against many gram-positive bacteria but a much lower activity against gram-negative bacteria. The side-chain, however, may confer other properties on the molecule that are of medical interest. Thus, penicillin V is more stable to acid than penicillin G (Brandl & Margreiter, 1954) and reaches higher levels in the blood when taken by mouth, while penicillin O is tolerated by patients who are sensitive to penicillin G. Cephalosporin N is much more hydrophilic than the other penicillins shown in Table I and has a very different range of activity, being much less active in vitro against gram-positive bacteria but more active against certain gram-negative bacteria, in particular the salmonellae.

All the penicillins so far described appear to be inactivated readily by the enzyme penicillinase. The antibiotic cephalosporin C (which, like cephalosporin N, is produced by a species of *Cephalosporium* and contains a side-chain derived from D-α-aminoadipic acid) is insensitive to penicillinase although it is both an inducer of the enzyme (Pollock, 1957) and a competitive inhibitor of the action of the enzyme on the penicillins (Abraham & Newton, 1956b). This relative of the penicillin family differs from the true penicillins in the β-thiolvaline fragment of the molecule (Abraham & Newton, 1956a; Abraham, 1957).

e. Bacitracins

The bacitracin family of polypeptide antibiotics is produced by *Bacillus licheniformis*. Bacitracin A, the main component of the family, may have the structure (VII) (Abraham & Newton, 1958; Craig, Konigsberg & Hill, 1958). Bacitracin B appears to be a mixture of peptides in which valine can



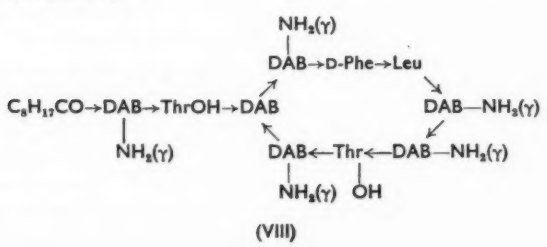
(VII)

replace the isoleucine of (VII) at various positions in the chain. The large ring in bacitracin A could be formed by condensation of the carboxyl group of an aspartic acid residue with the ε-amino group of a lysine residue in a dodecapeptide, and the *N*-terminal thiazoline-containing fragment of the molecule could be formed from an *iso*leucylcysteinyl sequence.

The effect of bacitracin A on *Staph. aureus* shows some striking similarities to that of penicillin (Abraham & Newton, 1958). The integrity of the labile thiazoline ring in the molecule is probably essential for activity. When the antibiotic is treated with hot dilute acid, which opens the thiazoline ring, or when it is kept in neutral aqueous solution, which results *inter alia* in the oxidation of the thiazoline to a thiazole, activity is lost. The configuration at the asymmetric centre of the *N*-terminal residue in bacitracin A also appears to be important. Racemization at this centre may be responsible for the reduction in activity which occurs when the peptide is kept in weakly acidic media at room temperature.

f. Polymyxins

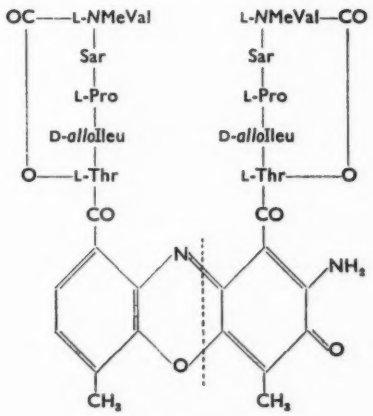
These antibiotics comprise a family of basic polypeptides formed by various strains of *Bacillus polymyxa* (Brownlee, 1949). Polymyxin B_1 may have the structure (VIII) (Hausmann, 1956). A residue of the uncommon αγ-diaminobutyric acid (like lysine in bacitracin A) is engaged in the formation of a ring from part of the peptide chain. An *N*-terminal residue of αγ-diaminobutyric acid is acylated by 6-methyloctanoic acid. *Iso*octanoic acid is found in this position in polymyxin B_2.



(VIII)

g. Actinomycins

Streptomyces antibioticus and other species of *Streptomyces* produce a large family of bright red actinomycins whose members contain the same phenoxazone chromophore linked to different peptides. Certain members of this family have been labelled C_1, C_2 and C_3 and the structure (IX) has been proposed for actinomycin C_3 (Brockmann, Bohnsack, Franck, Gröne, Muxfeldt & Süling, 1956). In other actinomycins, including those labelled I–V, D-*allo*isoleucine is replaced by D-valine (Bullock & Johnson, 1957).



(IX)

Analogy with the phenoxazones found in the ommochrome pigments of the eyes of insects suggests that the chromophore of the actinomycins is formed from two molecules of a 3-hydroxyanthranilic acid which may itself be derived from tryptophan. The two peptide fragments in a given member of the family may or may not be identical. This fact could be accounted for by the random coupling of two 4-methyl-3-hydroxyanthraniloyl peptides, as indicated by the broken line in (IX) (Johnson, 1956). The peptides contain a large lactone ring, the *C*-terminal carboxyl group being condensed with the hydroxyl group of a threonine residue, and in this respect they resemble another polypeptide antibiotic, etamycin (Sheehan, Zachau & Lawson, 1958).

Quantitative and qualitative changes in the mixture of actinomycins synthesized by a given species of *Streptomyces* are produced by changes in the amino-acid composition of the fermentation medium. In particular, it appears that the proline residue can be replaced by sarcosine, hydroxyproline, 4-ketoproline or pipecolic acid (Katz & Goss, 1958). There is some indication that the ratio of antitumour activity to general toxicity may be raised by changes in the peptide portion of the molecule.

h. Tyrothricin

A paper by Dubos on tyrothricin, which appeared in 1939, was the first of the modern publications on antibiotics. Tyrothricin, produced by *Bacillus brevis*, consists of two families of macrocyclic peptides, the neutral gramicidins and the basic tyrocidines. Tyrocidine A, a cyclic decapeptide, differs from tyrocidine B in containing an L-phenylalanine residue in place of an L-tryptophan residue (Craig, 1956). Gramicidin S, also produced by a strain of *B. brevis*, is a

cyclic decapeptide which contains the repeated amino-acid sequence L-Pro.L-Val.L-Orn.L-Leu.D-Phe (Consden, Gordon, Martin & Synge, 1947; Craig, 1956). The same sequence comprises one-half of the molecule of tyrocidine A.

2. Antibiotics Derivable mainly or partly from Acetate

Collie (1907), Robinson (1955), Birch (1957) and others have pointed out that certain structural features in a natural product suggest that the product is formed from a poly-β-keto acid (or an equivalent structure) and thus from acetate:

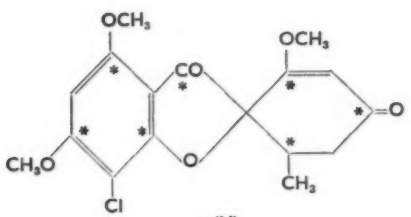
$$2x\ CH_3CO_2H \longrightarrow x\ (-CH_2.CO.CH_2.CO-)$$

These features include the presence of oxygen on alternate carbon atoms, or of unsaturated groupings obtainable from this distribution of oxygen by dehydration. The addition of extra groups, such as methyl, may be required to complete the molecule.

A number of the antibiotics used in medicine have structures of this type. In some cases the importance of acetate in their formation has been established by the use of ^{14}C-labelled acetate; in others experimental evidence has still to be obtained.

a. Griseofulvin

This antifungal antibiotic produced by several species of *Penicillia* has the structure (X) (Grove, MacMillan, Mulholland & Rogers, 1952). Its entire skeleton may be formed from a poly-β-keto acid, since alternate carbon atoms (*) are labelled when the compound is produced in the presence of [1-^{14}C]acetate (Birch, Massy-Westropp, Rickards & Smith, 1958). The methyls of the methoxyl groups and the chlorine atom are added by separate processes.



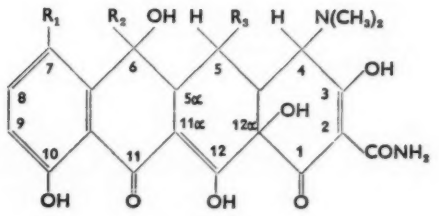
(X)

b. Tetracyclines

This family of antibiotics is formed by certain species of *Streptomyces*. The various members of the family, some of which are shown in Table II, can be represented by the general structure (XI) (Hochstein, Stephens, Conover, Regna, Pasternack, Gordon, Pilgrim, Brunings & Woodward, 1953). [1-^{14}C]Acetate and [2-^{14}C]acetate are good precursors of labelled tetracyclines (Snell, Wagner & Hochstein, 1956; Miller, McCormick & Doerschuk, 1956), and reduction of 5α(11α)-dehydrotetracycline may be the final step in the biosynthesis of the molecule (McCormick, Sjolander, Miller, Hirsch, Arnold & Doerschuk, 1958). The amount of 7-chloro- or 7-bromotetracycline formed, relative to other tetracyclines, depends on the halide content of the culture medium. 6-Demethyltetracyclines are synthesized by a mutant of *Streptomyces aureofaciens* (McCormick, Sjolander, Hirsch, Jensen & Doerschuk, 1957).

TABLE II. Nature of some Tetracyclines Produced by *Streptomyces* spp.

Compound (see formula XI)	R_1	R_2	R_3
Tetracycline	H	CH_3	H
7-Chlorotetracycline (aureomycin)	Cl	CH_3	H
5-Oxytetracycline (terramycin)	H	CH_3	OH
6-Demethyltetracycline	H	H	H



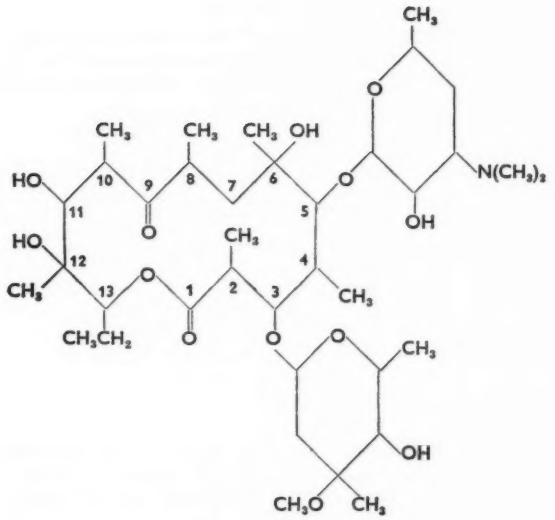
(XI)

The properties of the compounds listed in Table II show that the substituents R_1, R_2 and R_3 on the upper periphery of (XI) may all be varied without great effect on the activity of the molecule in vitro against *Staph. aureus*. 6-Deoxytetracyclines, obtained from tetracyclines by hydrogenolysis, also have a high activity (Stephens, Murai, Rennhard, Conover & Brunings, 1958). Total synthesis of (±)-dedimethylamino-12α-deoxy-6-demethylanhydrochlorotetracycline and the preparation of the (+) form of this compound from 6-demethylchlorotetracycline have shown that activity is not eliminated by removal of the dimethylamino group (Boothe, Kende, Fields & Wilkinson, 1959). The 6-demethyl- and 6-deoxytetracyclines are much more stable to acid than are other members of the family. The introduction of an aminomethyl group on to the amide nitrogen, by treatment of tetracyclines with formaldehyde and an amine such as morpholine or pyrrolidine, yields compounds that are active in vitro and readily soluble in water (Gottstein, Minor & Cheney, 1959).

Other changes in substituents, such as the conversion of the $CONH_2$ group to CN, result in a loss of activity. Further, the configurational requirements for activity appear to be strict. 4-*Epi*tetracycline and 5-*epi*tetracycline, which differ only in their configuration at C_4 and C_5 respectively from the natural products, have less than 5% of the activity of the latter against *Staph. aureus*. Dehydrogenation at $C_{5\alpha(11\alpha)}$ also results in a large loss of activity (McCormick, Miller, Growich, Sjolander & Doerschuk, 1958).

c. The Erythromycin (Macrolide) Group

Erythromycin (XII) is perhaps the best known of a group of antibiotics produced by *Streptomyces* spp. that consist of large lactone rings (called macrolides) linked glycosidically to novel dimethylamino sugars and, in some cases, sugars without an amino group (Gerzon, Flynn, Sigal, Wiley, Monahan & Quarck, 1956). Erythromycins B and C are found in the mother liquors from the crystallization of erythromycin. Among other members of the group are



(XII)

oleandomycin (Els, Celmer & Murai, 1958), the spiramycins (Paul & Tchelitcheff, 1957), and the magnamycins or carbomycins (Woodward, 1956). Table III shows some of their chemical properties.

Erythromycin B differs from erythromycin in having no OH at C_{12} of the macrolide core, while erythromycin C has the same core as erythromycin itself but a different sugar in the place of cladinose. Magnamycin B differs from magnamycin in having a double bond in place of an epoxide ring at C_{14-15} in the macrolide core.

In general, the structures of the large lactone rings of the erythromycin group of antibiotics suggest that these macrolides are formed by condensation of acetate and the addition of extra oxygen and methyl groups to the resulting unbranched carbon chains. The macrolide of erythromycin itself, however, has methyl groups attached to all the even-numbered carbons and this has raised the question whether it is formed, not from acetate and one-carbon fragments, but from propionate (Woodward, 1956). The experimental evidence is incomplete, but it appears to favour the view that acetate is the biogenetic unit.

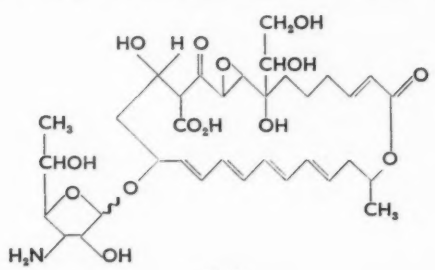
TABLE III. Some Antibiotics of the Erythromycin (Macrolide) Group

Substance	Molecular formula	Sugars obtained on hydrolysis	
		dimethylamino sugar	neutral sugar
Erythromycin	$C_{37}H_{67}O_{13}N$	Desosamine ($C_8H_{17}O_3N$)	Cladinose ($C_8H_{16}O_4$)
Erythromycin B	$C_{37}H_{67}O_{12}N$		
Erythromycin C	$C_{36}H_{65}O_{13}N$		Unnamed ($C_7H_{14}O_4$)
Oleandomycin	$C_{35}H_{61}O_{12}N$	Desosamine	Oleandrose ($C_7H_{14}O_4$)
Magnamycin	$C_{42}H_{67}O_{16}N$	Mycaminose ($C_8H_{17}O_4N$)	
Magnamycin B	$C_{42}H_{67}O_{15}N$		
Spiramycin A	$C_{45}H_{78}O_{15}N_2$	Mycaminose ($C_8H_{17}O_4N$) and another ($C_8H_{17}O_3N$)	Mycarose ($C_7H_{14}O_4$)
Spiramycin B	$C_{47}H_{80}O_{16}N_2$		
Spiramycin C	$C_{48}H_{82}O_{16}N_2$		

The similarity in structural pattern of the different antibiotics of the erythromycin group of macrolides is reflected in a similar range of antibacterial activity and in the fact that bacteria which have acquired resistance to one member of the group in vitro are also resistant to others. Erythromycin itself appears to be more effective against *Staph. aureus* than the other macrolide substances that are used in medicine, but it is clear that certain variations are possible in the size of the lactone ring, in the substituents on the ring, and in the sugars attached to the ring, without a major change in biological properties. The hydroxyl group in the desosamine fragment of erythromycin and oleandomycin can be esterified with acetic or propionic acid to yield active compounds that give superior blood levels (Stephens & Conine, 1959; Celmer, 1959). However, substitution of one of the *N*-methyl groups of erythromycin by hydrogen, propyl or *n*-butyl greatly reduces antibacterial activity (Flynn, Murphy & McMahon, 1955).

d. Polyene Antibiotics: Nystatin and Amphotericin

A group of antifungal antibiotics produced by *Streptomyces* spp. show ultra-violet absorption spectra characteristic of conjugated polyenes. Nystatin ($C_{46}H_{77}O_{19}N$) contains a tetraene, and amphotericin B ($C_{46}H_{73}O_{20}N$) contains a heptaene. Both substances are amphoteric and consist of C_{40} structures linked to the same amino sugar, mycosamine (Walters, Dutcher & Wintersteiner, 1957). Pimaricin ($C_{34}H_{49}O_{14}N$), another antifungal polyene, has recently been shown to consist of mycosamine linked to a large lactone ring containing conjugated double bonds (Patrick, Williams & Webb, 1958). Its structure (XIII) thus shows a similar



(XIII)

architectural pattern to that of the erythromycin group of macrolides, and it may be that nystatin and amphotericin B are compounds of a similar type.

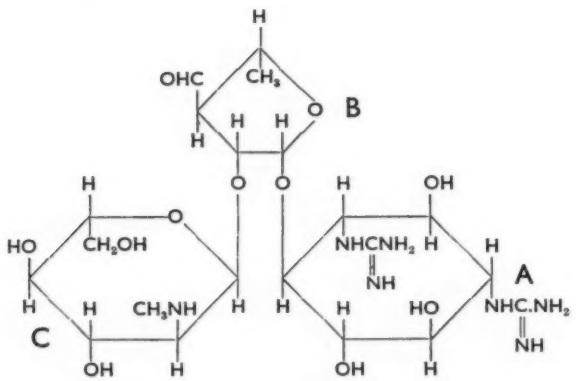
3. Antibiotics Derivable from Sugars

It has been suggested that the sugars attached to the macrolide antibiotics may be synthesized, like the large lactone rings, from acetate, but this view is not supported by a report that the labelled carbon of [1-^{14}C]acetate is incorporated only into the lactone ring of erythromycin and not into cladinose or desosamine (Vaněk, Majer, Babický, Liebster, Vereš

& Doležilová, 1958). Several sugar-like antibiotics produced by *Streptomyces* spp., in any event, are formed from other units. For example, the complete carbon skeleton of streptomycin is synthesized from D-glucose, but [^{14}C]acetate is only incorporated into the guanidyl side-chains (Hunter, 1956). Certain other basic antibiotics, kanamycin and the neomycins, show chemical similarities to streptomycin.

a. Streptomycin

This antibiotic (XIV) consists of three components, streptidine (A), streptose (B) and *N*-methyl-L-glucosamine (C), linked glycosidically (Kuehl, Peck, Hoffhine, Peel & Folkers,



(XIV)

1947). Mannosidostreptomycin, in which a residue of D-mannose is attached by a glycosidic linkage to C_4 of the *N*-methyl-L-glucosamine fragment of (XIV), and hydroxystreptomycin, in which the methyl group of streptose is replaced by a hydroxymethyl group, are also produced by *Streptomyces* spp. (Fried & Titus, 1948; Grundy, Schenck, Clark, Hargie, Richards & Sylvester, 1950).

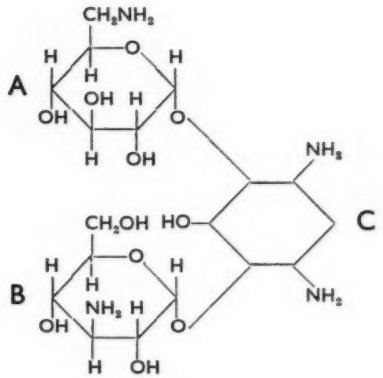
Certain compounds which react with the aldehyde group in the streptose fragment of streptomycin, such as cysteine, bring about inactivation. The aldehyde group can be reduced catalytically, however, with the formation of an active dihydrostreptomycin. The dihydro derivative, like streptomycin, has an ototoxic action but, unlike the latter, it appears to affect hearing more than equilibrium.

b. Kanamycin

The molecule of this substance (XV) is made up of 6-amino-6-deoxy-D-glucose (A) and 3-amino-3-deoxy-D-glucose (B) linked glycosidically to 2-deoxystreptamine (C). The latter differs from the streptidine of streptomycin in containing amino groups in place of guanidyl groups and in containing hydrogen in place of a hydroxyl group at C_2 (Cron, Evans, Palermiti, Whitehead, Hooper, Chu & Lemieux, 1958; Ogawa, Ito, Kondo & Inoue, 1958).

c. Neomycin B and C

These closely related antibiotics contain four fragments joined by glycosidic linkages. A diaminohexose (neosamine B or C)



(XV)

linked to D-ribose comprises a part of the structure known as neobiosamine B or C. The latter is joined to neamine, which is composed of a diaminohexose and 2-deoxystreptamine. The two antibiotics thus differ only in the neosamine fragment of the molecule (Rinehart & Woo, 1958).

4. Miscellaneous Antibiotics

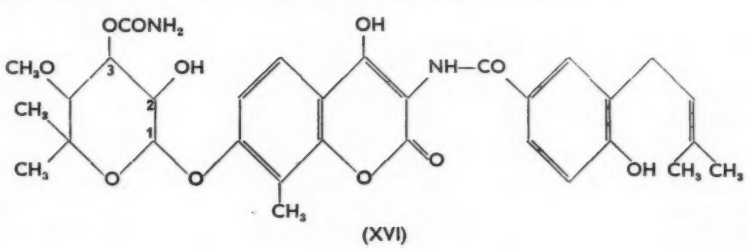
The structures of several of the antibiotics used in medicine have not been revealed in detail, or may not fall readily into any one of the groups already considered.

a. Novobiocin

Novobiocin is produced by two species of *Streptomyces*. The structure of this antibiotic (XVI) can be dissected into a new sugar, noviose, a substituted coumarin, and a substituted benzoic acid (Walton, Rodin, Stammer, Holly & Folkers, 1958). The enolic group of the coumarin imparts acidic properties to the molecule. At pH 10 novobiocin partly isomerizes to *iso*novobiocin, the carbamyl group of noviose migrating from C_3 to C_2. This change results in the loss of about one-third of the original activity (Hinman, Caron & Hoeksema, 1957).

b. Vancomycin

This antibiotic is produced by *Streptomyces orientalis*. It is an amphoteric substance, with a molecular weight of 3,200–3,500, which appears to contain amino and phenolic groups and carbohydrate. It yields D-glucose and aspartic acid, among other products, on hydrolysis (McCormick, Stark, Pittenger, Pittenger & McGuire, 1956).



(XVI)

c. Ristocetin A and B

These are produced by *Nocardia lurida*. They are amphoteric substances containing amino and phenolic groups and carbohydrate (Philip, Schenck & Hargie, 1957).

5. Conclusion

It has not been possible here to describe the methods by which organic chemists have determined the structures of many important antibiotics and achieved, in some cases, their total synthesis. This elegant work has provided the material in which biogenetic patterns are now beginning to appear. Certain types of hitherto unusual structures, such as macrolides, occur so often among the antibiotics that they may reflect broad peculiarities of the biochemical organization in micro-organisms. But the detailed mechanisms by which such structures are formed have yet to be elucidated.

Antibiotics with very different structures may evidently have a similar range of antimicrobial activity. Chloramphenicol and the tetracyclines provide an example. Members of the same structural family presumably have the same mode of action and often resemble each other, as might be expected, in their range of activity. In some cases, however, variations in structure which leave an essential nucleus of the molecule intact result in considerable changes in relative activity against different organisms, possibly because they affect the facility with which the compound makes contact with its site of action. Pharmacological properties may also be influenced by such structural variations. Examples of these situations are seen in the effects of different side-chains on the penicillin nucleus, and it is conceivable that new penicillins of medical interest will be prepared by the synthetic methods that are now available.

Nevertheless, the scope for improving the chemotherapeutic properties of antibiotics by chemical manipulations appears to be strictly limited. Although it may be possible to obtain a series of active compounds by varying the substituents attached to a given nucleus, a change in the structure of the nucleus itself, or in configuration at its asymmetric centres, is liable to be accompanied by loss of activity. The specific conformation of this portion of an antibiotic molecule, which presumably enables it to interact at several points simultaneously with a sensitive microbial structure, is normally essential for its antimicrobial properties.

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THE NATURE OF THE SELECTIVE TOXICITY OF ANTIBIOTICS

E. F. GALE Sc.D. F.R.S.

Medical Research Council
Chemical Microbiology Research Unit
Department of Biochemistry, University of Cambridge

1 Cell wall synthesis and the action of penicillin
  a Early studies on the action of penicillin
  b Composition of bacterial cell walls
  c Consequences of removal of the cell wall
  d Actions of penicillin
2 Membrane permeability and the action of surface-active antibiotics
3 Protein synthesis and the action of chloramphenicol
4 The future
References

An antibiotic is a substance which is synthesized by one organism and is toxic to other organisms. The substance thus displays selective toxicity and may differentiate between organisms which are potentially useful and others which are potentially harmful. Antibiotics that will inhibit the growth of a pathogenic organism without affecting the well-being of its host are of immense therapeutic importance and economic consequence. Many hundreds of antibiotics have been isolated during the last twenty years, but few have proved to have the type and degree of selective toxicity that has led to their use as chemotherapeutic agents. A knowledge of the nature of this selective toxicity is necessary for any future rational development of chemotherapy, and desirable for economic guidance of the industry which has sprung up around the production of antibiotics. Unfortunately, knowledge of the mode of action of antibiotics has lagged far behind their discovery, so that their existence provides a challenge to biochemists who study fundamental processes of life. Since the processes which are attacked by the antibiotics are clearly essential to the life of the sensitive organisms, investigation of the nature of these processes has, as a by-product, the promise of throwing light on the basic biochemistry of life and of pointing to the biochemical differences between sensitive and non-sensitive organisms.

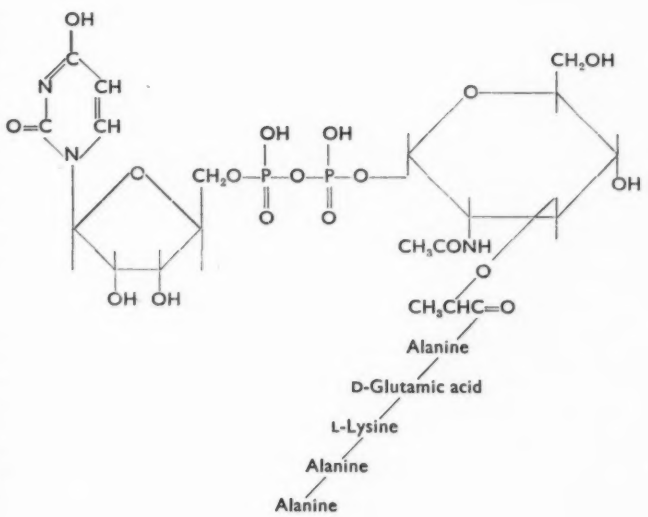
It is a reasonable guess that a selective inhibitor reacts with a substance specific to the sensitive organism, or inhibits a reaction peculiar to the parasite. Identification of such specific components or reactions has, however, been long delayed but, although it is still not possible to give a complete account of the action of any antibiotic in clinical use, investigations of the last few years have begun to point the way to the site of action of some of the "older" antibiotics and to enable a general description to be given of the nature of their selective action. The answers are gradually becoming apparent as a result of the coming together of investigations on the structure and metabolism of bacteria on the one hand, and of experiments planned to reveal inhibitory actions of antibiotics on the other. Promising results have been obtained with penicillin, polymyxin and chloromycetin (chloramphenicol) and, although many other selective antibiotics are under active investigation, it is proposed to restrict the present paper mainly to these three.

1. Cell Wall Synthesis and the Action of Penicillin

a. Early Studies on the Action of Penicillin

Penicillin has proved to be the most selective of known growth inhibitors. It is highly toxic to most gram-positive bacteria, markedly less effective against many gram-negative bacteria, and without obvious effect on animal, plant, protozoal and fungal cells. Some of the earliest investigations showed that the lethal effect of penicillin occurred only when sensitive cells grew in the presence of the antibiotic, and that cells of abnormal size and shape frequently formed before the lethal action was complete (Gardner, 1940; Hobby, Meyer & Chaffee, 1942; Hirsch, 1943–44; Hobby & Dawson, 1944; Chain & Duthie, 1945).

The biochemical finding which, after a delay of some years, pointed to what is probably the most important lesion in the cell treated with penicillin, was the observation by Park & Johnson (1949) that new phosphate-containing substances accumulated within *Staphylococcus aureus* growing in the presence of penicillin. Three of these substances were isolated by Park (1952): they all contained uridine diphosphate and *N*-acetylmuramic acid; one contained alanine, and another alanine, glutamic acid and lysine. Formula I was tentatively assigned to the most complex of the three substances. The optical specificity of the amino acids was unusual in that the glutamic acid was present as the D-isomer, while both D- and L-isomers of alanine were present. The function of these "Park nucleotides" was unknown until 1957, when Park and



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Strominger pointed out that they were related in structure to components of the staphylococcal cell wall.

b. Composition of Bacterial Cell Walls

Investigations of the chemistry of bacterial cell walls were made possible by the observation of Dawson (1949) that shaking suspensions of bacteria with minute glass beads in a sonic oscillator results in separation of the walls from the rest of the cell material in such a way that the walls can be centrifuged out of the broken material and isolated in a clean condition. Contaminating cytoplasmic substances can be removed by appropriate treatments, and the general method has now been applied to a wide variety of bacteria, with the result that we now know the chemical composition of the wall substance in many cases. Walls from different organisms differ markedly in composition. Walls removed from gram-negative organisms contain 11–20% lipid and, on hydrolysis, liberate a complete range of amino acids; walls from gram-positive bacteria contain less lipid (2–4%) and liberate a smaller number of amino acids (as few as four in some cases), which do not include the aromatic amino acids (Salton, 1956). In general the walls are composed of mucopolysaccharide and, again, the carbohydrate composition varies with the organism. These variations in composition are such that Cummins & Harris (1956) have suggested that they can be used as criteria for purposes of classification. Gram-positive walls have certain components in common: glucosamine, *N*-acetylmuramic acid, D-glutamic acid, alanine and either lysine or diaminopimelic acid (Cummins & Harris, 1956). The ratio of D-glutamic acid to *N*-acetylmuramic acid is 1 : 1. Park & Strominger (1957) pointed out that muramic acid, D-glutamic acid, alanine and lysine are present in both the staphylococcal cell wall and in the "Park nucleotide" and that the relative amounts are the same in both structures. Consequently they suggested that penicillin interferes with the synthesis of wall substance, and that the "Park nucleotides" might correspond to precursors of wall substance whose polymerization has been inhibited by the presence of the antibiotic. Baddiley and his co-workers have shown the presence of another major component in the walls of certain gram-positive bacteria; this is a polymer of ribitol phosphate which accounts for all the phosphate and 40–60% of the dry weight of the wall substance. Alanine and glucosamine are associated with the ribitol polymer in some organisms, and the complex has been called "teichoic acid" (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). In *Staph. aureus* treated with penicillin, cytidine diphosphate ribitol has been found to accumulate and this may bear a relationship to the teichoic acids of the wall similar to that of the "Park nucleotides" with the muramic acid complex (Buchanan, Greenberg, Carss, Armstrong & Baddiley, 1958).

c. Consequences of Removal of the Cell Wall

The bacterial cell wall is a rigid structure of considerable tensile strength. In certain species, e.g., *Bacillus megaterium*, *Micrococcus lysodeikticus*, chemical bonds in the wall substance are susceptible to the action of lysozyme, an enzyme found in egg-white and natural secretions such as sweat, saliva and tears. The walls of such organisms are completely dissolved by incubation with lysozyme, and incubation of suspensions of the organisms with the enzyme results in complete lysis. If, however, the osmotic pressure of the suspending medium is raised by the addition of high concentrations of sucrose, sodium chloride or other non-penetrating solutes, then the enzyme dissolves the cell wall and releases the naked cell body or protoplast (Weibull, 1953). The protoplast has most, if not all, of the metabolic and biosynthetic activities of the intact cell (McQuillen, 1956). It is, however, extremely sensitive to osmotic lysis and can be released in an intact state only if the osmotic pressure of the medium is of the same order as that of the cell contents. The protoplast is enclosed within a membrane which is impermeable to many metabolites. The concentration of these metabolites may be as high as 500 times that in the external medium, and the osmotic pressure within the cell may be as great as 20–25 atmospheres in a gram-positive coccus or 4–5 atmospheres in a gram-negative bacillus (Mitchell & Moyle, 1956b). The robustness of the intact bacterial cell, as opposed to the osmotic sensitivity of the protoplast, is due to the protection afforded, and the strength imparted, by the cell wall. Consequently any impairment of the wall structure or inhibition of its synthesis during growth will result in exposure of the membrane to the difference between internal and external pressures, and the consequent damage may culminate in lysis.

d. Actions of Penicillin

If penicillin acts by affecting the formation of wall substance, then death of the cell may be attributed to lysis of the exposed protoplast. If this is so, it should be possible to stabilize the cell in the presence of penicillin by addition of sucrose to the growth medium. Lederberg (1956) first showed that *Escherichia coli* can grow in the presence of penicillin if Mg ions and 10% sucrose are added to the medium and that such growth results in the formation of large, spherical and other abnormally shaped cells which are osmotically sensitive. Removal of the penicillin is followed by resumption of wall synthesis in some of these structures, which then grow out as normal bacillary forms.

Other effects of growth in the presence of penicillin have been described during the last fifteen years. The ability of staphylococci to assimilate amino acids from the medium is impaired and eventually abolished by addition of penicillin to the medium during growth (Gale & Taylor, 1947b; Brown & Binkley, 1956), and the antibiotic prevents the formation of inducible β-galactosidase in suspensions of *Staph. aureus* (Creaser, 1955). Amino acids are assimilated by active transport across the protoplast membrane, and β-galactosidase is a membrane enzyme in this organism. Consequently the question arises whether the impairment of these activities is a separate effect of penicillin or a consequence of damage to the membrane. Hancock (1958) has shown that the addition of 1 M-sucrose or sodium chloride to the suspending medium protects the cells from inhibition of either amino-acid transport or β-galactosidase synthesis in the presence of penicillin; the inhibitions that occur in the absence of a stabilizing agent must therefore be secondary effects. Hancock & Park (1958) have developed a method whereby the wall fraction can be separated from staphylococci and have been able to confirm directly that penicillin inhibits the incorporation of amino acids into the peptide portion of the wall substance.

The bacterial cell wall is a specific structure, differing in composition from the walls or membranes of other cells, and its synthesis therefore provides an excellent site for selective action. The wall substance is complex and it would be surprising if penicillin were the only agent to act in some way

upon the wall or its synthesis. Abraham (1957) found that bacitracin gives rise to accumulation of "Park nucleotides" in *Staph. aureus*, and Gale & Folkes (1953) had shown that bacitracin and penicillin had similar effects on the incorporation of amino acids and the development of certain enzymes in this organism. Park (1958) has now confirmed that bacitracin—and also oxamycin—inhibits the incorporation of amino acids into the wall peptide; chlortetracycline (aureomycin) at high concentrations inhibits the incorporation of amino acids into both wall peptide and cell protein but at bactericidal concentrations inhibits incorporation into cell protein only.

The early observations of the action of penicillin can now be explained. The antibiotic is effective against growing cells, since osmotic sensitivity is displayed only when growth takes place in the absence of a properly constituted cell wall, and the abnormal forms of cells represent the intermediate stages occurring during the development of osmotically sensitive protoplasts (or spheroplasts in which the wall is attenuated rather than completely absent) from normal cells.

2. Membrane Permeability and the Action of Surface-Active Antibiotics

As pointed out above, the cell body is enclosed within a membrane which poses an osmotic barrier to the free diffusion of many metabolites. Substances such as amino acids, purines, pyrimidines, phosphate and phosphate esters are concentrated within the cell, and the enzymes responsible for metabolism of such substrates are acting in concentrations markedly higher than those found in the normal external environment. Hotchkiss (1944) first showed that treatment of bacteria with tyrocidine or certain detergent substances resulted in release of nitrogen- and phosphate-containing substances from the cells, and that the cells were then nonviable although enzymic activities might continue at a very low rate. Among the substances released from the cells are the amino acids, purines, pyrimidines and phosphates which are normally concentrated inside the cells (Gale & Taylor, 1947a; Salton, 1951). Tyrocidine is too toxic to be of clinical use but certain other, less toxic antibiotics are now known to act in a similar fashion. Polymyxin, which can be used for disinfection of burns or grafts contaminated with *Pseudomonas pyocyanea* (Jackson, Lowbury & Topley, 1951), releases soluble constituents from sensitive gram-negative organisms. The site of action of such agents is the protoplast membrane. This has been proved by Newton (1956), who prepared a fluorescent form of polymyxin and demonstrated its fixation by the membrane in sensitive cells. The membrane is a lipoprotein structure (Mitchell & Moyle, 1956a; Gilby, Few & McQuillen, 1958; Weibull & Bergstrom, 1958) and the action of polymyxin upon it can be antagonized by metallic ions; a quantitative study of the metal antagonism indicates that polymyxin combines with phospholipid components of the membrane (Newton, 1954). Membranes from different cells differ in the amount of phospholipid they contain, but methods for the characterization of small amounts of phospholipid have not yet attained sufficient accuracy for it to be known whether the nature of the phospholipids varies in different cells. It seems probable that such differences form the basis of the selective action of polymyxin.

The protoplast membrane is not simply a passive semipermeable membrane. Since the membrane is impermeable in "resting" cells to nutrients such as amino acids which nevertheless accumulate to high concentrations within growing or metabolizing cells, it is clear that the membrane must possess active transport mechanisms, at least. There is also evidence that enzymes form part of the protein of the membrane (Mitchell & Moyle, 1956a, 1956b) and that the membrane may be concerned not only in the synthesis of protein (Beljanski & Ochoa, 1958; Butler, Crathorn & Hunter, 1958; Spiegelman, 1959; Connell, Lengyel & Warner, 1959) but also of the cell wall (Crathorn & Hunter, 1958). The studies of Rowley, Cooper and their colleagues (see review by Cooper, 1956) on the fixation of radioactive penicillin by bacteria indicate that penicillin reacts with lipoprotein material almost certainly derived from the cell membrane, so that the site of action of penicillin may be a synthetic mechanism in the membrane.

3. Protein Synthesis and the Action of Chloramphenicol

Chloramphenicol is without action on the synthesis of cell wall material (Hancock & Park, 1958; Mandelstam & Rogers, 1958) and does not release soluble constituents from sensitive cells. A survey of the various metabolic systems that can be studied as separate events within the cell has shown that the only process which is inhibited by chloramphenicol at growth-inhibitory concentrations is the synthesis of protein (Gale & Folkes, 1953). The syntheses of protein and ribonucleic acid (RNA) are normally closely associated in biological systems, and chloramphenicol is of interest in that it prevents protein synthesis without having any obvious inhibitory action on RNA synthesis. However, evidence has accumulated during recent years that the RNA which is formed in the presence of the antibiotic is markedly different from that formed in its absence. The RNA formed under normal conditions is very stable and consists mostly of macromolecular material of molecular weight 10^5 and higher. The nucleic acid formed in the presence of chloramphenicol is very heterogeneous and mostly composed of material with molecular weight 5,000–10,000; it is highly unstable and its accumulation in the presence of the antibiotic is due to rapid synthesis and breakdown, the former process being faster than the latter. If the chloramphenicol is removed, synthesis stops and the nucleic acid already formed breaks down to nucleotides and other degradation products (Neidhardt & Gros, 1957; Pardee, Paigen & Prestidge, 1957; Horowitz, Lombard & Chargaff, 1958; Gale, 1958).

Recent studies by Hoagland (1959) of the nucleic acids of mammalian tissues have revealed that there are two forms of RNA present: a "soluble" form of relatively small molecular weight and the macromolecular form associated with the ribonucleoprotein particles of the microsomal fraction. Both forms are believed to play a role in protein synthesis; the soluble material reacts with activated amino acids to produce labile, reversible complexes, and peptide bond formation between amino acids appears to take place in or on the ribonucleoprotein particles. Both species of RNA can be demonstrated in bacteria, and the RNA that accumulates in the presence of chloramphenicol more closely resembles the soluble form than the macromolecular form. It is, for example, heterogeneous in composition, of variable but small molecular weight, and forms labile, reversible complexes with activated amino acids (Pardee *et al.* 1957; Gale, 1958; Lacks & Gros, 1959). The work of Hoagland, Zamecnik &

Stephenson (1957) has suggested that the amino-acyl complexes of soluble RNA represent an early stage in protein synthesis and that the soluble RNA may act as a carrier of amino-acyl residues from the activation site to the ribonucleoprotein particles where peptide bond formation takes place. If a similar sequence of events takes place in bacteria, it would appear that chloramphenicol inhibits some stage in the sequence, a stage occurring after the formation of amino-acyl-soluble RNA complexes but before peptide bond formation. The site of this inhibition is still a matter for speculation; it is, of course, possible that one or more of the soluble RNA complexes formed in the presence of the antibiotic is abnormal.

Chloramphenicol is a selective inhibitor in that it prevents the growth of many bacteria but is without significant effect on the growth of yeast, fungal, protozoal or animal cells. The resistance of these cells may be attributable to their possession of membranes through which the antibiotic cannot pass. There have been few reports of any investigations of the action of chloramphenicol on amino-acid incorporation in cell-free mammalian systems, and such reports as are published show indefinite results. It seems that, if chloramphenicol does inhibit incorporation in such preparations, then it does so only at concentrations markedly higher than those which are completely inhibitory in bacteria. Protein synthesis is a process common to all living cells, but our knowledge of the mechanism is far too elementary for us to state whether this mechanism is the same in all cells. The existence of chloramphenicol and its selective action on bacteria may point to the existence of differences in the mechanisms of protein synthesis in bacteria and other cells.

Protein synthesis is undoubtedly a most complex process and must offer many different sites for inhibition. Eliminative metabolic surveys similar to those undertaken in the case of chloramphenicol have also indicated that the tetracyclines inhibit protein synthesis at growth-inhibitory concentrations (Gale & Folkes, 1953). A further similarity is that the tetracyclines do not inhibit the formation of RNA, but the RNA produced is again of heterogeneous nature with molecular weight smaller than normal (Cascio, personal communication, 1959). Saz & Slie (1954a, 1954b) have found that the aromatic nitro-reductase of certain bacteria is inhibited by low concentrations of chlortetracycline and that this inhibition is released by Mn ions. This has led to the suggestion that this antibiotic acts by virtue of its affinity for metal ions, removing them from sites where they are essential for the activation of enzymes. There has not, so far, been any demonstration of reversal by metals of the inhibition of protein synthesis by chlortetracycline. No basis for the selective action of chlortetracycline has yet been proposed.

4. The Future

Biochemical investigations of antibiotics are undertaken in the belief that an understanding of the mode of action should lead to the more rational development and design of chemotherapeutic agents. It has to be admitted that empirical screening procedures have been far more successful than any development stemming from fundamental investigations. This is because the antibiotics of importance all have actions on basic biochemical reactions of which we have little or no knowledge. In fact, the antibiotics have proved of far greater use as tools for metabolic investigations than have the investigations themselves proved to be as guides to better drugs. Fortunately it does not follow that this will continue to be the case, even in the reasonably near future. For example, once we can see why a relatively simple molecule like chloramphenicol should stop the transfer of amino-acid residues from soluble RNA complexes to ribonucleoprotein particles, then a new era of design of inhibitors will be opened up. Most of our ideas concerning inhibition of vital processes stem from the discovery of Woods (1940) that sulphonamides act by competitive inhibition of the metabolism of the structurally related metabolite, *p*-aminobenzoic acid. A vast literature on the design and possible action of "metabolite analogues" has arisen since that time (for a general treatment see Woolley, 1952). Attempts have frequently been made to interpret the actions of antibiotics along the same lines. Thus it has been suggested that chloramphenicol can be regarded as an analogue of the amino acid phenylalanine (Woolley, 1950), but convincing evidence that such a relationship underlies its action has yet to be produced. However, the action of chloramphenicol in the context of the soluble RNA complex may lead us to reconsideration of this hypothesis. Should it prove to react as, or compete with, an amino acid in this system, then we shall learn much about the design of really effective analogues. If it does not act in this way, then we may see our way to a new basis for chemotherapeutic interference. And we shall certainly learn much about the biochemistry of protein synthesis, and possibly about comparative aspects of the process, as a very useful side-line.

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DRUG RESISTANCE AND MECHANISMS FOR ITS DEVELOPMENT

M. R. POLLOCK M.B.

National Institute for Medical Research
Mill Hill, London

1 Mechanisms of drug resistance
  a Insusceptible metabolism
  b Impedance to uptake, or penetration, of drug into cell
  c Destruction of drug
2 Emergence of drug-resistant populations
  a Acquisition of resistance by individual cells
  b Heritability of drug resistance
  c Population changes: natural selection
  d The great controversy
3 Practical applications
References

Progress of research into the problem of resistance to antibiotics is held back by lack of knowledge of the biochemical mechanisms by which these drugs act. Fortunately, it is possible to study some of the mechanisms of drug resistance, even at a biophysical or biochemical level, without necessarily knowing very much about how the drug works—just as it is possible to study (at least at a biological level) the question of how micro-organisms become resistant, without understanding the biochemical basis of the resistance mechanism itself. Research can proceed along all three lines to some extent independently, despite the fact that they are so closely interrelated.

In an article as short as this must be, the subject of drug resistance can be dealt with only in the barest outline. For more detailed reviews and discussions of the subject the reader is referred to some of the more recent publications, each of which, in its own particular way, deals with the problem more comprehensively than can be attempted here (Schnitzer & Grunberg, 1957; Bryson & Szybalski, 1955; Cavalli-Sforza & Lederberg, 1953; Davies & Gale, 1953; Wolstenholme & O'Connor, 1957).

1. Mechanisms of Drug Resistance

There are three main mechanisms by which an organism may resist the inhibitory effects of a drug. (i) A drug-sensitive reaction essential for growth in susceptible strains may be either absent or supplemented by one that performs the same function and is unaffected by the drug. (ii) The surface properties of the cell may be such that the drug cannot penetrate. (iii) The cell may possess a means of destroying the drug before it exerts its effect. Obviously, these mechanisms are not mutually exclusive and differences between sensitive and resistant organisms are not absolute; it is usually a question only of degree.

a. Insusceptible Metabolism

This may be the most frequent and most important mechanism of resistance, the understanding of which depends most closely upon knowledge of how the drug acts.

i. A metabolite essential for the growth of both resistant and sensitive organisms may be synthesized by a chain of reactions not involving one that is inhibited by the drug. Streptomycin-resistant *Escherichia coli*, for instance, appears to be able to dispense with a streptomycin-sensitive reaction responsible for the formation of an intermediate in citric acid production (Umbreit, Smith & Oginsky, 1951; Umbreit, 1953), though it is not yet certain that inhibition of this reaction is in fact the means by which streptomycin inhibits growth. It is also possible to by-pass a susceptible, essential reaction by providing the necessary end-product in the medium; this would appear to be what may be happening (though probably indirectly) when organisms are found to be much more resistant if grown in one particular medium rather than another—e.g., *E. coli* or *Staphylococcus aureus* growing anaerobically are far more resistant to streptomycin than when grown in the presence of O_2 (Lightbown, 1957).

ii. A metabolite whose incorporation into some compound essential for growth is competitively inhibited by the drug in the sensitive strain may be formed in such large quantities that it can overcome the inhibitory effect of the drug. This would appear to be the basis for some cases of sulphonamide-resistance in staphylococci (Landy, Karkum, Oswald & Streightoff, 1943), where greatly increased quantities of the sulphonamide antagonist, *p*-aminobenzoic acid (PAB), are formed by the resistant strains.

iii. An essential enzyme or enzyme system, inhibited by the drug in the sensitive strain, may be present in a form which renders it insusceptible to inhibition. For example, Saz & Martinez (1956) found that a partially purified cell-free nitroreductase preparation from aureomycin-resistant *E. coli* was less inhibited by aureomycin than was a similarly prepared extract from sensitive cells, and Hotchkiss & Evans (1958) have produced evidence suggesting that sulphonamide-resistance shown by certain strains of pneumococci depends directly upon the ability to form modified enzymes (concerned in folic acid synthesis) with increased relative affinity for PAB. But, so far, in no case have the relevant enzymes been isolated from both sensitive and resistant strains in a pure state and their properties compared, though work on other systems suggests that qualitatively altered enzymes can occur through mutation.

b. Impedance to Uptake, or Penetration, of Drug into Cell

i. Resistance to Drug Penetration

Early work (see Schnitzer & Grunberg, 1957) on the reaction of trypanosomes to dyes, arsenical compounds (tryparsamide) and acriflavine suggested that the commonest basis of resistance was inability of the drug to penetrate into the cell. More recently, isotope incorporation experiments have shown that ^{14}C-labelled isoniazid was taken up freely into the cells of isoniazid-sensitive strains of *Mycobacterium tuberculosis*, whereas there was negligible uptake of the drug by insensitive organisms (Barclay, 1953). Chloramphenicol-resistance shown by a strain of *Pseudomonas fluorescens* (derived from a sensitive parent strain) would similarly appear to be due to the development of some mechanism for preventing the drug from entering the cell, since chloramphenicol

inhibited succinate oxidation in extracts of resistant and sensitive cells, and in intact sensitive cells, but had little effect on the reaction in intact resistant cells (Kushner, 1955).

ii. Masking or Absence of Drug Receptor

The original drug-receptor idea of Ehrlich is probably best instanced by the case of penicillin-sensitive staphylococci which have been shown to fix specifically and irreversibly small quantities of penicillin. The quantity of penicillin fixed by different strains of varying penicillin-resistance is roughly proportional to their penicillin-sensitivity: the more resistant a strain is, the less penicillin-receptor substance appears to be present (see Cooper, 1956).

c. Destruction of Drug

The only really clear-cut case of drug resistance being due to a specific drug-destroying enzyme is that of penicillinase, which in a number of cases—especially amongst the naturally occurring penicillin-resistant staphylococci and in the *Bacillus* genus (Bondi & Dietz, 1948; Spink & Ferris, 1945; Pollock, 1957b)—has been conclusively proved to be a factor determining the penicillin-resistance of the organisms that produce it (see also Table I). Similarly, the "chloramphenicol reductase" activity of a chloramphenicol-resistant strain of *E. coli* was found to be three times higher than that of the normal sensitive strain (Merkel & Steers, 1953), though it is difficult to believe that this alone could account for the 10,000-fold difference in chloramphenicol-resistance between the two strains.

TABLE I. Relation between Penicillin-Resistance and Rate of Penicillinase Synthesis in Strains of *Bacillus cereus*

Strain	Penicillin-resistance*	Rate of penicillinase formation†
5 (parent strain)	0.01	15
5/P (mutant)	0.3	9,500
5/B (mutant)	3.0	75,000

* Expressed as maximum concentration of penicillin, in units/ml., allowing colony formation from at least 50% of viable spores inoculated into nutrient agar.

† Expressed as mean number of molecules formed per cell. (Penicillinase formation in these strains is entirely "constitutive", i.e., takes place without need for induction by penicillin.)

2. Emergence of Drug-Resistant Populations

"Drug resistance" in micro-organisms seems to be a term too often indiscriminately applied to individual cells and to populations of cells without carrying any consistent implication as to its heritability. It can be argued that the "organism" in microbial infections is not the single cell, but a population of cells. Certainly, individual bacteria behave very differently according to whether they are alone or alongside a large number of others. Moreover, it is difficult to study the behaviour of single micro-organisms and practically all experimental work on bacterial metabolism is done on large populations. Microbiologists have an unfortunate habit of talking and writing (and probably thinking) mainly about individual micro-organisms whilst working almost exclusively with huge populations. No doubt these factors have contributed largely to the misunderstandings that have arisen. From a strictly biological and biochemical point of view, the properties of the cell itself must be considered first; the extent to which a character such as drug resistance is handed on to daughter cells, and the composition and properties of the population as a whole, can then be more logically tackled afterwards.

This method of approach is not the one usually adopted in papers on this subject. If it is accepted, two quite separate questions must be asked.

i. How does the individual cell acquire resistance?

ii. To what extent, and by what means, is the property of resistance passed on to daughter cells?

a. Acquisition of Resistance by Individual Cells

There are numerous plausible speculations as to how a cell might increase its powers of resisting the effects of a drug. From a biochemical standpoint they probably all involve changes in the activity or rate of production of enzymes. In general, the types of change known from direct experimental evidence to be involved appear to fall into two main groups, where different mechanisms (not mutually exclusive) are operative. (i) A specific increase in the rate of formation of an enzyme responsible for by-passing or eliminating the effects of the drug on the cell, directly or indirectly due to the action of the drug itself (i.e., by the process known as enzymic induction—see Pollock, 1959). (ii) A change in the genic constitution of the cell leading to the formation of new, or increased amounts of, enzymes which determine drug resistance. In both groups, changes in ability of the drug to penetrate the cells are included, since it has been shown that cell permeability can change (adaptively) in a manner analogous to the changes involved in enzymic induction *sensu stricto* (see Cohen & Monod, 1957).

i. Enzymic Induction

The possible relationship of this phenomenon to drug resistance is most clearly illustrated by work on the induction of penicillinase formation in *Bacillus cereus* by treatment of cells with penicillin (Pollock, 1957b). The effect of the penicillin is almost instantaneous, and even very small doses of the antibiotic will stimulate cells to increase their rate of enzyme formation up to three hundred times, with concomitant increase in their penicillin-resistance. There is good (though admittedly indirect) evidence, that, under most conditions (as in other cases of enzymic induction), all or most of the cells respond equally to the effects of the inducer. Many strains of staphylococci respond likewise (Geronimus & Cohen, 1957). As far as other drugs are concerned little is known, but it is reasonable to suppose that analogous changes may take place.

ii. Genic Alterations

Many characters involving the ability to synthesize certain enzymes in micro-organisms appear to be controlled by entities similar to the genes studied intensively in higher organisms. In *E. coli* these gene-like entities, some of which are concerned with specific resistance to drugs (see Cavalli-Sforza & Lederberg, 1953), are known to be arranged in a definite linear order along the chromosome, and their positions and reassortment and alterations can be followed, by a number of different techniques, with very great precision. It is likely that similar

chromosomes exist in other bacteria. There are two main ways in which the gene composition of a bacterial cell may be altered: (i) an individual gene may be altered or lost by "mutation"; (ii) the order and/or composition of the total gene complement may be changed by recombination of elements derived from two different cells.

Gene mutations occur very rarely. Usually, as far as any single gene is concerned, only one in every 10^6–10^{10} new cells undergoes a mutation. Although the primary change itself is sudden, it may take several hours before it is reflected in demonstrable alteration to the cell's metabolic function ("phenotypic lag"). It may involve quite small changes (e.g., ability to grow in M/500 sodium azide, Lederberg, 1950), or relatively large leaps in drug resistance (e.g., 1,000-fold increase in streptomycin-resistance of *E. coli*, Newcombe & Hawirko, 1949). Really big increases in drug resistance appear often to need a series of successive mutations, each involving relatively small jumps (e.g., in the development of chloramphenicol-resistance in *E. coli*, Cavalli & Maccacaro, 1952). Most gene mutations occur "spontaneously" (i.e., independently of any known factors in the environment), though the mutation rate can be increased by irradiation and by certain chemical compounds, such as the N and S mustards, peroxides, urethane, maleic anhydride and others (see Westergaard, 1957) and even, perhaps, by antibiotics themselves (e.g., streptomycin, Wilson, 1950). According to available evidence, the type of mutation thus induced, though sometimes affected by the type of mutagen employed, does not seem to bear any relation to the physiological effects of the inducing agent. Thus in so far as streptomycin, for example, may increase the total number of mutations, it does not appear to cause a specific increase in the proportion of mutations towards streptomycin-resistance. The difficulty in establishing this very important fact arises from the impossibility of testing the drug resistance of a cell without allowing contact between cell and drug, which, therefore, might be held to have itself caused the mutation. That gene and gene-like mutations towards drug resistance can occur before the drug reaches the cell has now been demonstrated by means of two different types of technique. (i) The Luria & Delbruck (1943) "fluctuation test", and modifications of the same principle (Newcombe, 1949), have shown that very small cultures grown from the same inoculum give very widely varying numbers of drug-resistant cells (as indicated by numbers of colonies growing on drug-containing plates inoculated with samples from the different cultures). This would be expected if the mutations occurred at random very infrequently, since a late mutation (e.g., one occurring only two generations before the sample was taken) would yield only four resistant cells in the whole culture, whereas an early mutation (e.g., one occurring eight generations before sampling) would yield 2^8, i.e., 256 resistant cells per culture. If the drug itself caused the mutation, and on the assumption that all cells were equally susceptible to change, numbers of resistant cells in the different cultures would vary very little (only according to a Poisson distribution). (ii) The replica plating method (Lederberg & Lederberg, 1952) and its variants (e.g., Cavalli-Sforza & Lederberg, 1956) is simply a technique for sib (or rather cousin) sampling (e.g., for drug resistance) from a very large number of clones in order to subculture selectively from those clones which show "family characteristics" of drug resistance, without allowing the drug to gain access to the cells. It has thus been possible to produce a homogeneous culture of streptomycin-resistant cells of *E. coli* which have never been in contact with streptomycin (Lederberg & Lederberg, 1952). A possible objection that this may only be a method for selecting cells which are susceptible to a direct mutative effect of the drug (which has, of course, to be used finally in order to demonstrate that the selected cells are indeed resistant) has been met by Sneath (1955) who could demonstrate the effect of the mutant character (production of extracellular penicillinase) in the culture supernatant of the *B. cereus* 5/B mutant strain without ever exposing the cells to penicillin.

Gene recombination can occur in a number of ways. Transfer of genetic material from one cell to another may result from sexual union, certainly in *E. coli* and possibly in other organisms (see Lederberg, 1955; Wollman, Jacob & Hayes, 1956), by carry-over on an infecting, non-virulent bacteriophage previously grown in the donor cell, i.e., "transduction" (Zinder & Lederberg, 1952), or by direct penetration of genetically specific deoxyribonucleic acid (DNA) in pneumococci and *Haemophilus*, i.e., "transformation". This genetic transfer is known to involve the transmission of the character of drug resistance from cells of one strain to those of another (e.g., transfer of penicillin- or sulphonamide-resistance between strains of pneumococci (Hotchkiss, 1951; Hotchkiss & Evans, 1958); and of streptomycin-resistance between strains of *E. coli*), but would not by itself be expected to lead to an increased total number of resistant cells. It is conceivable, however, that from the reassortment of genes which thereby occurs drug-resistant recombinants might arise from sensitive parent cells, owing to the synergistic action of genes (brought together in the process) which, acting separately, confer no property of resistance. This has not yet, in fact, been observed, but has not apparently been much looked for. Sexual crosses between different types of chloramphenicol-resistant strains of *E. coli* yield recombinants with a range of different degrees of chloramphenicol-resistance but so far none with increased resistance (Cavalli & Maccacaro, 1952).

b. Heritability of Drug Resistance

Gene changes are, by definition, extremely stable. They are also, by definition, part of the self-perpetuating apparatus of the cell which is reproduced at every cell division. Thus, in the absence of any intervening factors, the altered cell characters for which they are responsible will be passed on regularly to daughter cells at each generation. However, reverse mutations (e.g., from drug resistance to drug sensitivity) and reassortment of genes (due to further gene transfer from one cell to another) may occur quite frequently, though still usually affecting only a minute proportion of the total population. For example, back-mutation rates from streptomycin-dependence to streptomycin-sensitivity have been measured in *E. coli* (Demerec, 1951; Bertani, 1951). Although back mutations from drug resistance to sensitivity do not appear to have been the subject of much study, it is difficult to believe that they do not occur at the same order of frequency as the numerous other reverse mutations (mainly relating to nutritional deficiencies) studied in bacteria generally. Thus the processes involved in genic change are not absolutely irreversible.

Enzyme induction, on the other hand, does not, as far as is known, involve the gene apparatus of the cell. A cell induced to form penicillinase in large quantities by treatment with penicillin is not thereby changed to the extent of being able to pass on the property of increased penicillinase formation

(and therefore increased penicillin-resistance) to its progeny indefinitely. The induced character is not, however, immediately lost when the cells are subsequently grown in the absence of the inducing agent. In organisms reproducing themselves, like bacteria, by binary fission there need be no loss of cell material on reproduction and in most cases of enzyme induction the induced enzyme is stable and merely "dilutes out" in the expanding mass of protoplasm when the cells grow in the absence of the inducer (see Pollock, 1958). With penicillinase induction in *B. cereus*, the effect of the inducer is more pronounced in that it appears to activate a stable enzyme-forming system (EFS) which continues to function at constant rate, indefinitely, even when the penicillin is withdrawn. The EFS itself is "diluted out", not just the enzyme, so that the induced character persists (though with decreasing magnitude) to a detectable extent for up to ten cell generations. Nothing, however, is yet known about how the induced character (whether it be penicillinase itself or the penicillinase-forming system) is distributed amongst the dividing cells on subsequent growth without inducer. Induced β-galactosidase appears to be partitioned equally amongst the cells during reversion (Benzer, 1953), but where a specific EFS itself is concerned it is rather more likely that the number of independently functioning systems within each cell is very limited and that therefore some segregation will occur early on during growth. The various possibilities are illustrated schematically in fig. 1 where the numbers of molecules per cell of a drug-destroying enzyme (which may be taken as proportional to the resistance of the individual cell) are indicated according to whether only the enzyme or an EFS is induced by the drug and whether the induced character

FIG. 1A

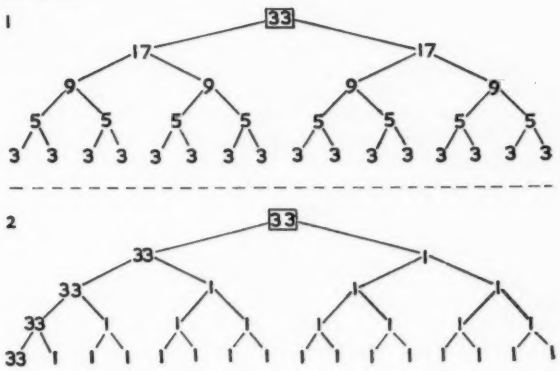

FIG. 1B

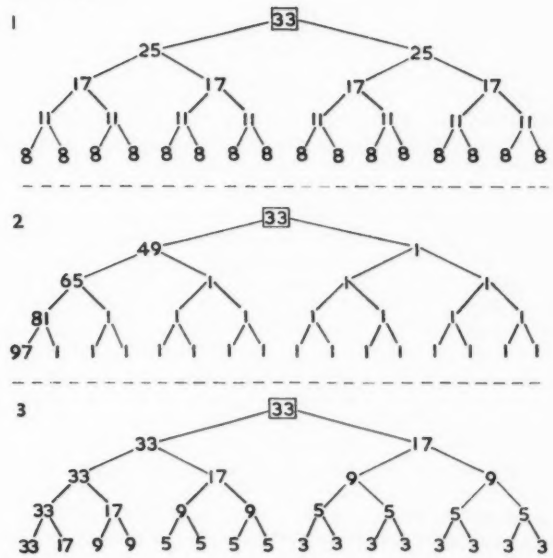

FIG. 1C

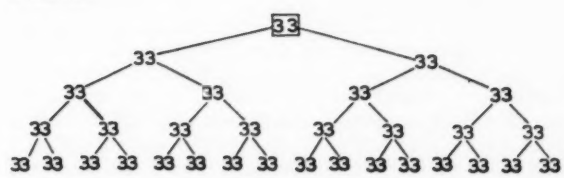

FIG. 1. Scheme to Illustrate Possible Types of Inheritance of Drug Resistance in Bacterial Clones, each being Derived from a Single Resistant Parent Cell (enclosed in square thus: [33]) Multiplying in a Drug-Free Medium.
The individual levels of resistance are given by numbers indicating the quantity of a drug-destroying enzyme present in each cell

The resistance of the original sensitive strain (before development of resistance) is taken to be 1 unit of enzyme per cell.

A. Resistance acquired by accumulation of 32 extra units of enzyme induced by drug in resistant parent cell

1. Equal partition of preformed enzyme amongst cells on cell division
2. Complete retention of all preformed enzyme by one of the pair of daughter cells formed at each cell division (i.e., complete segregation)

B. Resistance acquired by accumulation of 32 extra units of enzyme and activation of system(s) forming 16 extra units of enzyme every cell generation, induced by drug in resistant parent cell

1. Equal partition of enzyme and enzyme-forming system (EFS) amongst cells at each cell division
2. Complete segregation of enzyme and EFS at each cell division
3. Complete segregation of EFS, but equal partition of enzyme at each cell division

C. Resistance acquired by gene mutation conferring ability to produce 32 extra units of enzyme in each cell as it is formed on division of parent cell

is completely segregated in one cell on cell division, or is equally partitioned amongst all daughter cells. The points to appreciate are: (i) some measure of drug resistance will persist after removal of the drug in all cases; and (ii) if maximal segregation occurs (fig. 1A(2), 1B(2), 1B(3)) there will persist one fully resistant cell in the population for every cell originally induced; this cell cannot give rise to a whole colony of resistant cells, of course, but it will have survived, and if the drug is subsequently removed would grow into a colony of (sensitive) cells having the property of the "persisters" described by Bigger (1944). A possibly analogous type of "limited inheritance" in bacteria is the transmission of ability to produce flagella in cells of a certain abortively transduced culture of *Salmonella typhi-murium* studied by Stocker (1956). Other instances of what has been called "linear inheritance" (especially in protozoa where individual cells can be directly studied with ease) have been reviewed by Lederberg (1956).

Figure 1C illustrates the situation where resistance is due to a gene mutation and therefore results in the "classical" type of stable inheritance involving transfer of the new character to all cells in the clone through an indefinite number of cell generations.

c. Population Changes: Natural Selection

So far we have considered only changes in individual cells. Neither of the two types of alteration discussed is, by itself, sufficient to account for the development of homogeneous cultures of hereditarily stable drug-resistant cells that we know can arise from sensitive cells after in-vivo or in-vitro "training" by growth in the presence of the drug. Gene mutations to drug resistance, though hereditarily stable, affect only a minute proportion of the cells in a population, and, so far as is known, are not specifically provoked by the presence of the drug. Such mutations will not lead to the development of drug-resistant cultures without some selection pressure to favour more rapid growth of drug-resistant cells. This is naturally most efficiently provided by the drug itself. A relatively very small difference between the growth-rates of mutant and parent cells in the presence of the drug is sufficient to cause a complete (or very nearly complete) switch from a sensitive to a resistant population (e.g., the development of penicillin-resistance in a strain of *B. cereus* studied by Sneath, 1955), as long as growth in the presence of the drug is allowed to continue for some time; the smaller the difference in growth-rates, the longer the period of training necessary for complete conversion to occur (see Bryson & Szybalski, 1952). An analogous reverse population shift, operated by selection pressure, from drug resistance to drug sensitivity may occur through back mutations followed by growth without the drug, under which conditions the drug-sensitive cells frequently grow more rapidly than the resistant variants (see Mitchison, 1953; Cavalli-Sforza & Lederberg, 1953).

Similarly, but for a different reason, enzyme induction, of a type that confers increased drug resistance, does not by itself lead to any genetically stable conversion of the population to drug resistance, even though all the cells are affected in the first place. A suggestion (Pollock, 1953) of how a sequentially induced series of enzymes could theoretically lead to self-perpetuating cycles of biochemical reactions with genetic stability remains, so far, largely speculative. The role of enzymic induction in the development of heritable drug resistance may lie rather in the temporary breathing-space it can give cells in danger of annihilation by a drug, by allowing time for a stable mutation to appear and natural selection to complete the conversion to a genetically resistant population. Its contribution to the ability of the microbial species as a whole to withstand the drug must rest not only in the immediate effects of induction but also in the (albeit limited) prolongation of the effects (increased enzyme) after withdrawal of the drug, which will increase the chances of survival—at least of a fraction of the population—should the culture meet with a second onslaught. However, a recently described example (Novick & Weiner, 1957) of a stable, heritable increase in susceptibility to β-galactosidase induction (simulating a gene mutation) in *E. coli*, resulting from a single prior treatment with high concentrations of an inducer, suggests that the gap between specific enzyme induction and heritable transformation may not be as wide as might be at first supposed.

d. The Great Controversy

The two types of change at the cellular level leading to increased drug resistance discussed so far are clearly differentiated by the fact that the specifically induced change (enzyme induction) is not genetically stable, whereas the genetically stable change (gene mutation) does not appear to be specifically induced. The question arises as to whether any other type of change may play a part in the development of drug resistance. It would be very surprising if there were not other mechanisms, but so far evidence for their existence is slender. Basically the same question may be put in a form which is more familiar to bacteriologists: Can a specifically induced change (e.g., drug-induced drug resistance) ever be passed on to cell progeny as a stable heritable character? Or, alternatively: Can the type of gene mutation ever be specifically determined by environmental factors? Many claims have been made in favour of environmentally induced heritable transformations in micro-organisms. The two best-established cases — temperature-induced antigenic transformation in *Paramecium* (see Beale, 1954) and acriflavine-induced respiratory-deficient "petite" mutants in yeast (see Ephrussi, 1952) —involve non-genic cytoplasmic changes and do not appear to be adaptational in function. Neither example seems to be closely related to the problem of specifically induced drug resistance. Other claims (e.g., Linz, 1950; Dean, 1955; Kossikov, 1957) are open to the oft-repeated criticism that the experimental conditions employed have not been designed to eliminate the possibility of selection pressures operating in favour of mutant cells which may have originated independently of the presence of the drug (or other agent). Those workers who appear to be already convinced that environmentally induced heritable changes have been successfully demonstrated seem to base their opinion on the following kind of evidence, the truth of which—at least in certain cases —would probably not be contested by any worker in the subject. (i) The degree of resistance developed by in-vitro "training" procedures often bears a close relation to the concentration of drug used for the training (Eagle, Fleischman & Levy, 1952). (ii) Previously trained drug-resistant cultures undergo spontaneous reversion to drug sensitivity when grown in the absence of the drug (Barber, 1949). (iii) All the cells of an untrained culture, when inoculated on agar containing a new sole source of nutrient to which adaptation is necessary before growth will occur, may eventually produce colonies of fully adapted organisms (Dean & Hinshelwood, 1954b). (iv) The longer the training the more genetically stable is the

resistance developed (Dean & Hinshelwood, 1954a). (v) Training to complete drug resistance, or adaptation to a new carbon source, appears often to take place in suspensions of cells undergoing no net increase in total or viable count (e.g., Akiba, 1955).

It will, however, be apparent from what has previously been pointed out and from the following considerations that none of these arguments is conclusive. (i) Drug resistance can depend on the cumulative effects of a number of separate gene mutations (Cavalli & Maccacaro, 1952), and selection pressure at a given drug concentration would not be expected to operate in favour of a very highly resistant gene combination when a less highly resistant mutant will grow just as well. (ii) Back mutants to drug sensitivity are known to occur, and frequently grow more rapidly in the absence of the drug than do resistant mutants. (iii) Some multiplication of cells, and therefore mutations, followed by selection, may occur on solid media containing a new source of carbon, before colonies are macroscopically visible (Pollock, 1957a). (iv) Long-continued training in the presence of the drug may simply mean that the proportion of sensitive cells remaining in the culture has become so low that reversion of the culture to sensitivity, by selection when the drug is removed, takes a proportionally longer time. (v) Even when there is no net increase in numbers of cells, death (often followed by lysis) and regrowth of survivors may occur so as to allow mutations (which may anyway sometimes occur without cell multiplication—see Ravin, 1958) together with selection pressure to operate in favour of the emergence of a drug-resistant population.

On the other hand, there are no good *a priori* arguments against the existence of specifically induced heritable changes in micro-organisms. It is perfectly possible to conceive speculative schemes of how they may occur. Hinshelwood (1946, 1949) has indeed elaborated an internally consistent theory based on the interrelationships between enzymes in a steady-state system, which demonstrates how an automatic metabolic adjustment of the cell to the inhibitory effect of a drug on a single enzyme reaction could lead to a heritable change towards drug resistance. It is difficult to believe that some such scheme does not operate in some cases to some extent. The experimental evidence adduced in support of it, however, has so far been open to most of the objections listed above: it can equally well—in some cases more satisfactorily—be explained on a hypothesis based on spontaneous mutations followed by selection. That mutations to drug resistance can occur in the absence of the drug is now accepted—even by those (see Dean & Hinshelwood, 1957) who in the past have often argued against the validity and relevance of the tests designed to prove their existence (Hinshelwood, 1946, 1953). The question that remains is whether any other types of heritable change exist (particularly any directly provoked by the drug itself). It seems likely that this problem will not be settled until it is possible to do accurate work on the properties of single cells.

Yudkin (1953) has suggested a mechanism for development of drug resistance, which he has called "clonal variation with selection". This in fact amounts to the hypothesis that cells in a clone exhibit a finely graded series of individual levels of drug resistance, statistically grouped around the mean of the resistance of the original parent cell. Superimposed selection processes might then lead to the development of almost any level of drug resistance in the emerging population. This idea is not formally distinct from the assumption that mutations (of some sort) are always occurring and that any mutation might affect the drug resistance of the cell to a certain extent. This sort of phenomenon would not be expected to produce the big changes in drug resistance which are known to occur and which often take place in single jumps. Such evidence as there is, including that available from Yudkin's own work on proflavine-resistance (Thornley & Yudkin, 1959a, 1959b), suggests that the process he postulates probably accounts for only relatively minor variations in drug resistance.

3. Practical Applications

A rational basis for a practical approach to the problem of drug resistance would include attempts to (i) prevent the appearance of mutations and gene recombinations; (ii) reduce the selection pressures allowing mutants to multiply at the expense of the original sensitive cells (and increase selection in favour of the reverse process); and (iii) inhibit specifically the formation, and/or the functioning, of enzymes responsible for destroying the drug or providing alternative drug-resistant metabolic pathways. If progress has been limited, the way is at least open for further research along these lines. Antimutagenic agents are known to exist against chemical mutagens (Novick & Szilard, 1952) though they have so far been little exploited. Selection pressures in favour of drug-resistant mutants can be reduced by the avoidance of low, frequently administered, doses, and indiscriminate use, of antibiotics. The frequency of emergence of drug-resistant mutants will be greatly decreased by the simultaneous use of two unrelated antibiotics, since gene mutations occur independently of each other, and the chances of a double mutation (towards resistance against two drugs) occurring in the same cell will be the product of the chances against the appearance of a single mutation—i.e., in the region of 1 in 10^{12} or more cells, and thus extremely rare. Quite apart from any ordinary synergistic effect, the simultaneous use of two or more drugs may be encouraged also because one of the two may inhibit the metabolic pathway within the cell which is responsible for its resistance against the other. Clinical successes with combined antibiotic therapy certainly provide some justification for these purely theoretical arguments. "Trickle" dosing with minimal quantities of drugs is to be discouraged also because it might be expected to induce drug-destroying enzymes (and allow other forms of metabolic adaptation to occur) without being sufficient to inhibit completely all metabolic processes within the cells. There is also the hope that substances may be found that will specifically inhibit the formation of enzymes responsible for drug resistance. Such "enzymic repression" is known to occur in biosynthetic pathways of metabolism (see Pardee, 1959) and may be a general phenomenon. Temperatures above those optimal for growth have been found to inhibit penicillinase formation more than cell growth and thus decrease the penicillin-resistance of *B. cereus* (Knox & Collard, 1952). Even if this type of specific enzyme repression cannot be achieved there is the second-best possibility of finding specific inhibitors of enzyme function, such as cephalosporin C (Abraham & Newton, 1956) or a specific antipenicillinase antiserum (Perlstein & Liebmann, 1945; Tacking, 1955; Markov, 1956) against penicillinase.

It is fortunate that some, at least, of these measures might

be expected to work at a genetic level, at a populational level (by control of selection pressures) and at a purely physiological level within the cell. They may therefore prove to be successful in certain cases even though it is not yet known what mechanism for the development and expression of drug resistance is operative.

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THE PRINCIPLES OF THERAPEUTIC USE OF ANTIBIOTICS

LAWRENCE P. GARROD M.D. F.R.C.P.

Department of Pathology
St Bartholomew's Hospital, London

E. F. SCOWEN M.D. F.R.C.P.

Director, Medical Professorial Unit, Dunn Laboratories
St Bartholomew's Hospital, London

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The first decision to be made when antibiotic treatment is contemplated is whether it is necessary at all. Many of the more detached and thoughtful writers of recent years—some of whom are perhaps seldom faced with the task of actually treating a sick patient—have protested in strong terms against the abuse of these drugs for trivial purposes and their indiscriminate prescription with little idea of the precise purpose which they are likely to serve. Admittedly production figures, which now extend to hundreds of tons per annum in the United States of America, seem out of proportion to any likely demand for treating serious disease, and the study described by Nolen & Dille (1957) of consumption in a single small community furnishes exact data clearly betraying much unnecessary use. It is also noteworthy that, in many recorded instances of death from shock following a dose of penicillin, the indications for giving this appear to have been inadequate. Rosenthal (1958), describing 30 deaths from this cause in New York City, considered the indication for the fatal dose to have been adequate in 12 cases, doubtful in 13, and inadequate in 5, including a patient with a sprained toe. Among the diagnoses in 10 cases, 6 of which were fatal, reported by Cheng & Chiang (1956), were toothache, conjunctivitis, and the common cold.

It would be easy enough to conclude that antibiotics should never be prescribed for minor illnesses, but the problem is not so simple. What begins as a minor illness may develop into something more, particularly in certain predisposed patients. It would consequently be unwise to attempt the definition of hard-and-fast rules about conditions for which antibiotic treatment is justified. Each case must be judged on its merits, but if these are deliberately assessed, and if the treatment has an explicit rational basis, really indiscriminate use will thereby be excluded.

1. Bacteriological Diagnosis

It must be emphasized that the indication for treatment with an antibiotic is not usually a disease, but an infection due to a particular micro-organism. Naturally this distinction does not always apply; in typhus or typhoid, in syphilis, erysipelas, scarlet fever or carbuncle, the bacteriological diagnosis is implicit in the clinical. But in pneumonia and other infections of the air passages, in meningitis, in infections of the urinary tract and of wounds, and in puerperal fever, the cause of the infection must if possible be ascertained. The sulphonamides and antibiotics, with their peculiarly selective action on different bacterial species, have forced clinicians to think in bacteriological terms, and those who have best adapted their way of thinking have been the most successful in the use of these drugs. It is often desirable not only to know the cause of an infection, but to verify that this organism is normally sensitive to the drug which it is proposed to use. This has placed a heavy and constant responsibility on the laboratory, which has the task of furnishing this information by methods which reconcile speed with dependability.

2. Choice of an Antibiotic

Granting that a patient has an infection for which treatment with an antibiotic appears to be indicated—rather than, for instance, with a sulphonamide—the choice depends on several considerations, of which the primary one is the presumed or known activity of the antibiotic against the bacterial species causing the infection. To make even a preliminary choice on this basis calls for some knowledge of the range of activity of each of a number of drugs to which additions are still being made.

Table I is an attempt to present a general picture of the activity of the principal antibiotics against the commoner bacteria: it omits fungi and the antifungal antibiotics such as nystatin and amphotericin, the rickettsias and viruses. Absolute validity cannot be claimed for all the figures contained in it: they are derived from the findings of different workers using a variety of methods.

Gram-positive organisms are grouped in the upper part of the list of species and antibiotics active principally against them on the left of the vertical headings: the top left part of the table thus contains most of the information bearing on the treatment of infections due to streptococci, staphylococci and pneumococci. Some of these antibiotics have no useful action at all on any gram-negative species, except perhaps the highly vulnerable *Neisseria* and *Haemophilus*. Penicillin, on the other hand, partly because it has been more exhaustively studied than any other antibiotic, is known to be active in higher concentrations against some of the *Enterobacteriaceae*. These findings are not merely of academic interest: paradoxical as it may seem, penicillin may be the drug of choice for treating an *Escherichia coli* infection of the urinary tract (Scowen, Badenoch & Shooter, 1957), and does in fact kill this organism in urine almost as rapidly as streptomycin or neomycin (Garrod, 1959). Penicillin has also been successfully used in the treatment of typhoid fever (Boger, Schimmel & Matteucci, 1951). Synnematin B (cephalosporin N) is also effective in this disease (Benavides, Olson, Varela & Holt, 1955), and this antibiotic is a form of penicillin (Florey, 1955). Among other antibiotics in the group acting predominantly on gram-positive organisms, erythromycin has some action on several of the *Enterobacteriaceae*, probably of little clinical importance,

and novobiocin is active against *Proteus* only. Otherwise the lower left part of the table contains no entries.

In the last column on the right of the table is polymyxin, the activity of which is exclusively against gram-negative organisms: all the *Enterobacteriaceae* listed are sensitive to it, with the important exception of *Proteus*. The rest of the right-hand part of the table is occupied by broad-spectrum antibiotics, the tetracyclines, which are entered as one, since there are only minor differences between them, chloramphenicol, neomycin and streptomycin. These are the only antibiotics for which significant entries can be made for every, or almost every, bacterial species throughout both of the main classes, gram-positive and gram-negative. The only gaps denote the inactivity of streptomycin and neomycin against *Clostridia*. It is not suggested that all these figures indicate any possibility of therapeutic usefulness: neomycin and streptomycin, for instance, have a very poor action on streptococci, and *Pseudomonas pyocyanea* is relatively resistant to the whole of this group. So also is *Proteus* to some of them: its high degree of sensitivity to neomycin is noteworthy, since this compensates for the inactivity against this organism of polymyxin.

a. Indications for Sensitivity Tests

The values given in Table I are those normal for each species, but many of them are subject to the proviso that previous exposure to the antibiotic may have changed these values profoundly. Acquired bacterial resistance is already a serious obstacle to successful antibiotic treatment and an even graver threat to its future.

Fortunately there are some infections in which it need not be feared. They include all those, such as plague, tularaemia, anthrax and brucellosis, the only source of which is an animal reservoir where previous contact with the antibiotic is unlikely to have occurred. Secondly, even when such exposure takes place, the facility with which resistance can be developed is very variable, and there are instances in which it cannot be developed at all. From this point of view antibiotics fall into three distinct classes.

Penicillin, vancomycin and ristocetin are antibiotics in connexion with which this problem scarcely exists. The frequency with which staphylococci are now found to be resistant to penicillin may seem to belie this statement, but this is not in fact an example of acquired resistance: it is due to the dissemination of originally resistant strains while sensitive ones were being eliminated. In other species, penicillin-resistance is no problem: some organisms, such as haemolytic streptococci and pneumococci, cannot acquire it even under the most favourable conditions in vitro; others in fact have not done so during ten or more years of lavish use, as is clear from comparisons made by Finland and his colleagues of the sensitivity of strains isolated early and late in this period (Finland, 1958). This statement must be qualified by recording that a moderate degree of resistance to penicillin has begun to appear in gonococci (Cradock-Watson, Shooter & Nicol, 1958). Vancomycin is an antibiotic to which staphylococci are never resistant, nor can much resistance be artificially produced (Geraci, Heilman, Nichols, Wellman & Ross, 1956; Garrod & Waterworth, 1956). Ristocetin, evidently a closely similar substance, behaves in the same way.

At the other extreme is streptomycin, to which resistance of high degree can develop in almost any species so rapidly that it may nullify the effect of even a brief course of treatment in the individual patient. Resistance to erythromycin and to novobiocin is acquired rather less rapidly, but can have the same effect.

Lastly the tetracyclines and chloramphenicol are antibiotics to which any species can acquire resistance, but much more slowly, so that the result is not so much to interfere with the treatment of the individual patient as gradually to build up a more resistant race of organisms over a considerable period of time.

The methods by which bacterial sensitivity to antibiotics can be determined are dealt with elsewhere in this issue. Of the two main alternatives, the elaborate and time-consuming tube-dilution method is not often necessary except as a guide to a prolonged course of treatment, as in subacute bacterial endocarditis or tuberculosis. For most other purposes an agar-diffusion method is adequate, and its application in primary culture enables a result to be obtained in one day (see Plate I, A, B and Gould, Plate I, D in this number of the Bulletin). In properly prepared cultures interpreted by an experienced worker, such results are a trustworthy guide to treatment (Garrod, 1958).

b. Other Considerations Governing Choice

Although efficacy is the first consideration, other factors may influence the choice of an antibiotic. Perhaps the most serious of these concerns the liability of the antibiotic to cause undesirable side-effects. These are described in another contribution to this issue,[1] and it is only necessary to indicate briefly here the general policy which they seem to demand from us. Some antibiotics, including streptomycin, neomycin, chloramphenicol, bacitracin and polymyxin, are capable of exerting direct toxic effects. Some of these are rare and others relatively common: some depend on dosage and duration of treatment or an impairment of renal function, and others are unpredictable; but, regardless of any of these circumstances, another drug which never has such effects is preferable to one which can have, if the two are therapeutically equivalent. Superinfections, varying from trivial to fatal, are associated mainly with the broad-spectrum antibiotics, notably the tetracyclines: their efficacy in suppressing the normal flora of the alimentary tract and thus making way for invaders from without is easily comprehensible by reference to Table I. Thus in patients strongly predisposed to serious intestinal superinfections, particularly those subjected to major abdominal operations, these antibiotics are best avoided if possible.

Cost is a factor which should not be disregarded, whether the bill has to be paid by an individual or the State. Penicillin is much the cheapest of the antibiotics, and some of those in which manufacturers have monopolistic rights are unduly expensive. A course of treatment which is going to cost £25 should not be embarked on lightly, and that is a small sum compared with the cost of some long-continued forms of treatment, such as that of hepatic cirrhosis with neomycin or chronic bronchitis with one of the tetracyclines. It is usually held that economic considerations should not be allowed to influence the practice of medicine, but a situation may well arise, if indeed it has not already arisen, in which they must.

3. Choice of Preparation and Route

If systemic infection is successfully to be overcome, an effective concentration of the antibiotic must be attained at

[1] See Dunlop & Murdoch, p. 67 of this number of the Bulletin.—Ed

TABLE I. Sensitivity of Important Pathogenic Species of Bacteria to the Principal Antibiotics

	Normal minimum inhibitory concentrations (μg./ml.)											
	Penicillin	Erythromycin	Oleandomycin	Spiramycin	Novobiocin	Vancomycin	Bacitracin	Tetracyclines	Chloramphenicol	Neomycin	Streptomycin	Polymyxin
Staphylococcus pyogenes	0.012	0.12	0.5	2–4	0.12	1	2	0.12	8	1	2	
Streptococcus pyogenes	0.006	0.03	0.25	0.25	2	0.5	0.25	0.25	2	>128	32	
Streptococcus faecalis	5	0.5	2	2	0.12	1	50	0.5	2	128	64	
Streptococcus pneumoniae	0.006	0.03	0.25	0.25	1	0.5	10	0.25	2	128	64	
Clostridium welchii	0.12	2	16	64	32	1	4	0.03–0.25	4			
Bacillus anthracis	0.015	0.25	1	4	1	4		0.12	4	2	1	
Actinomyces israeli	0.06	0.1	0.25	2	2	2	5	1–4	1–4	10	4–64	
Neisseria gonorrhoeae	0.003	0.06	2	1	2	32+	5	1	1	10	5	1
Haemophilus influenzae	1–2.5	1	16	16	5			1	0.5	5	5	1
Mycobacterium tuberculosis								10	10	0.5	0.1	
Escherichia coli	20–>100	32						1	8	1	2	0.25
Klebsiella aerogenes								2	8	0.5	2	0.25
Klebsiella pneumoniae	2–100							0.5	1	0.5	1	0.12
Proteus spp.	5–100				4			40–>80	10	1	2	
Pseudomonas pyocyanea								20	200	50	50	0.12
Salmonella spp.	5–20	32+						1	2	0.5	8–16	0.12
Shigella spp.								0.5–2	1–8	1–4	2–4	0.12
Brucella abortus	20				5			2	2	4	0.5–4	2

The information in this table is derived in part from publications by other authors too numerous to quote, and in part from observations, published or unpublished, by one of us (L.P.G.).

Where no figure is given, either information is lacking or the organism is known to be highly resistant.

The values given are those normal for the species: higher degrees of resistance are common, particularly to some of the antibiotics, and in staphylococci and various gram-negative bacilli.

The expression "32+" means that some strains are inhibited by this concentration and others not.

the site of infection at the earliest opportunity and maintained until the active process is at an end. The mode of administration, either oral or parenteral, is primarily determined by the nature of the antibiotic, but in all suitable cases oral administration is to be preferred. This route has many practical advantages and it is easier to maintain an effective and more constant systemic level by frequent doses than by any other means except continuous intravenous administration.

The tetracyclines, chloramphenicol, erythromycin, oleandomycin and novobiocin are usually given by mouth, and effective systemic levels can be achieved and maintained by regular dosage. The character of the preparation used may materially influence the degree of absorption, and in some cases esterification, as with erythromycin propionate, may achieve more easy absorption than the use of the free base. In others the character of the excipient has to be considered.

The tetracyclines are absorbed much less well if exhibited with calcium salts, which clearly should be avoided in the excipient mixture.

In conditions of extreme urgency, or when oral administration is difficult, these antibiotics can be given parenterally. The intravenous route is to be preferred as it ensures an early and high concentration and is commonly used for tetracycline, erythromycin and novobiocin. Although intramuscular preparations are sometimes available, it is often difficult to maintain an effective systemic level, and this is particularly difficult in the case of novobiocin (Foltz, 1959).

Phenoxymethylpenicillin is given orally and is suitable for the treatment of mild and susceptible infection. "Soluble" (sodium or potassium) penicillin is also at times used by mouth and an effective level may be achieved; but the variability of absorption makes this unreliable, except at times in small children. This route is unsuitable for penicillin administration when infection is severe and a high level of penicillin is required. In these circumstances penicillin must be given intramuscularly, either as soluble penicillin, or as a mixture of soluble penicillin and the procaine salt. The use of procaine penicillin diminishes the frequency of injection which is required to maintain an effective level, though by its use alone it is not possible to obtain a high circulating level of penicillin.

The frequency of oral dosage is dictated by the rate of destruction or excretion of the drug. Each dose should be given so that adequate absorption occurs before the declining level of the previous dose falls below effective concentration. In most cases administration every six hours will suffice to ensure this, but no latitude is to be allowed in this time-factor without jeopardizing therapeutic efficiency.

Many antibiotics are not absorbed from the alimentary tract and in the treatment of systemic infection they must be given parenterally. Streptomycin is given by intramuscular injection, vancomycin and ristocetin by intravenous injection, and kanamycin may be given by intravenous or intramuscular routes. By the intravenous route these antibiotics may achieve an effective level with 12-hourly administration, though they need to be given well diluted and usually over a course of an hour or longer. To achieve an effective systemic level of streptomycin, it has to be given by intramuscular injection at least 12-hourly, although, in the long-term treatment of tuberculosis, injection on alternate days in combination with other drug treatment has been found adequate.

The failure of absorption from the intestinal tract does not prevent the antibiotic action in the lumen of the gut. It provides an excellent method of using high intestinal concentration for sterilization of intestinal infection without the hazards of systemic action. Neomycin, bacitracin, polymyxin and kanamycin are all used in this way without risk to ear or kidney.

All these antibiotics are excreted mainly by the kidney, the rate of excretion determining not only the frequency of dosage required but the concentration which will occur in the urine. This concentration also allows of a far higher level of antibiotic activity in the urine and urinary tract than is possible elsewhere in the body except by local injection. This property can be turned to advantage in the treatment of urinary infection. Antibiotics useless in the treatment of systemic infection by a given species may be suitable for the treatment of infection by the same organism in the urinary tract by virtue of the enormous concentration which can be achieved.

Conversely, impairment of renal function diminishes the excretion of the antibiotic, and caution is required in management. The use of standard doses may lead to excessive accumulation which may be dangerous or at best aggravate the risk of unpleasant sequelae.

The effective control of systemic infection assumes an adequate diffusion from the blood into the tissues. The drug may not enter serous exudates in adequate quantity, and direct injection into the cavity is necessary. Secretion into the lumen of the bronchial tree and the gut may not occur in adequate concentration and this may be of special importance in the treatment of chronic bronchitis and bronchiectasis.

The cerebrospinal fluid is notoriously exceptional. Although chloramphenicol traverses the choroid plexus in effective concentration, other antibiotics may not. When, however, the meninges are inflamed, the antibiotic may enter the fluid in the exudate and effective levels may be produced, though high concentrations in the blood are essential if this is to be achieved. In many circumstances it may be necessary initially to inject the antibiotic directly in the subarachnoid space if immediate and high concentration be indicated.

Direct instillation of antibiotic solutions into infected effusions in the pleura and in joint cavities is often necessary to ensure effective concentrations. Local instillation may also be necessary in the conjunctival sac, in the infected middle ear cavity by instillation or insufflation, and into the bronchial tree by atomization of antibiotic solutions.

4. Duration of Treatment

The duration of treatment is determined by the nature and response of the infection. Treatment must continue at effective levels until the infection has been eliminated. In general, treatment is continued until the patient has been apyrexial for at least 48 hours, but this general rule applies largely to acute diseases which lead to spontaneous recovery and ultimate complete resolution. It is in this group of diseases that the wider use of antibiotics is possible, for adequate bacteriostasis will usually be sufficient to enable the infection to be overcome, whereas in more chronic infection bactericidal efficiency is demanded if the disease is to be wholly eradicated.

The treatment of infections prone in their natural history to relapse, like typhoid fever, needs to be more prolonged, but persistence is even more necessary in the treatment of chronic infection. Though the lesions of syphilis may resolve with a few weeks' treatment with penicillin, six to eight weeks may be necessary to sterilize the lesions of subacute bacterial endocarditis; months may be needed to control the lesions of tuberculosis and to sterilize the kidney with chronic pyelonephritis.

The duration of treatment in these circumstances has been dictated by clinical observation but requires in each case direct demonstration that the process in the individual is at an end, by demonstrating that clinical activity is absent and that the blood remains sterile in bacterial endocarditis, and that the urine is free from pus and bacteria in chronic pyelonephritis. Arbitrary durations of treatment are only indications of average experience and not therapeutic laws.

Treatment designed to eradicate infection may be continued at lower levels, to prevent subsequent reinfection. Prolonged treatment with penicillin is used to prevent reinfection with *Streptococcus pyogenes* in rheumatism, and broad-spectrum antibiotics may be used in an endeavour to prevent relapse or exacerbation of chronic bronchitis.

To exploit fully the use of antibiotics, minute attention to the nature of the infection and the exhibition of the antibiotic is entailed, but care with the details of general management is imperative. Early supportive measures in severe infection, combined with the use of corticosteroids or the administration of γ-globulin, may enable the patient to survive long enough for the antibiotic to be effective.

5. Accessory Factors in Treatment

Antibiotic treatment sometimes fails because it cannot succeed alone, and other measures necessary to its success are neglected. It has been frequently reiterated that chemotherapy is not a substitute for good surgery, but only an adjunct to it, and many illustrations of this principle could be cited, of which the most obvious is failure to detect and drain a collection of pus. When infection is secondary to obstruction or stasis, in the urinary tract or elsewhere, the effect of any chemotherapy will be only temporary unless the underlying condition can also be relieved.

A special example of providing the conditions necessary for successful antibiotic treatment is the control of urinary pH. Streptomycin is about 50 times more active in an alkaline than in an acid urine within the normal range of variation, and its administration should never be begun for the treatment of a urinary tract infection until the urine has, if necessary, been rendered constantly alkaline. Neomycin is similarly affected: other antibiotics, including penicillin and chlortetracycline, are more effective in an acid medium. Another factor in this sphere of treatment is the volume of urine. Many patients undergoing surgical treatment are encouraged to drink enormous volumes of fluid and, in the resulting large output, an antibacterial drug may be diluted beyond the point of its effectiveness. Unless a diuresis is clearly indicated for some specific reason, it may be advisable actually to restrict fluids for a time in order to produce a maximal effective concentration of the drug in the urine (Garrod, 1959).

6. Observations of the Effects of Treatment

a. Repeated Bacteriological Examinations

It is often desirable to follow the effects of antibiotic treatment by repeated cultivation of material from the site of infection. If the causative organism persists, this may be an indication that an ineffective antibiotic is being used, that the dose is inadequate, or that some other necessary form of treatment has been overlooked. Naturally what is to be expected depends much on the site and nature of the infection: gonococci cease to be cultivable within three hours of an injection of penicillin, but tubercle bacilli may persist in the sputum for weeks or months during a course of treatment which finally achieves its object completely.

It seems useful here to draw a distinction between infections of body-surfaces, whether internal or external, which are normally populated by bacteria, and those of interior parts of the body which should be entirely bacteria-free. In the former, it may be enough merely to suppress the growth of a pathogenic organism: in the latter, the organism must be completely eliminated if recovery is to be assured. Thus in bacterial endocarditis every organism in the vegetations must be killed, and strictly speaking, repeated blood cultures during a course of treatment should be made to verify its effect. Actually this is often an unrewarding proceeding, because cultures are very rarely positive during treatment even when this is ultimately going to fail. Meningitis is another condition in which total sterilization of the affected area is essential, and every available specimen of cerebrospinal fluid should be cultivated to verify that this has been achieved. The same considerations apply to the urinary tract, with the added proviso that specimens should ideally be examined not only during and shortly after a course of treatment, but after a further and longer interval to exclude recurrence.

In the presence of suppuration or necrosis, rapid elimination of the organism is not to be expected: thus there is little to be gained by repeated cultures from such a lesion as a carbuncle. In infections of mucous surfaces, assuming that the clinical effects of treatment are satisfactory, the time for follow-up cultures is convalescence: thus penicillin treatment of tonsillitis or scarlet fever may or may not have eliminated the haemolytic streptococcus from the throat, and after any *Salmonella* or *Shigella* infection the stools should be cultivated repeatedly to determine whether a carrier state is persisting.

b. Antibiotic Assays in Body-Fluids

The concentrations which a given dose of any antibiotic should produce in the blood and other body-fluids are known, but it should not always be assumed that the normal concentration is being attained. There is some irregularity in absorption of any orally administered antibiotic, and if treatment by this route is not achieving its full effect, a determination of the amount of the antibiotic in the blood at an appropriate interval after a dose, or of the daily output in the urine, may yield significant results. In the treatment of meningitis it is highly desirable to verify that adequate concentrations are being attained in the cerebrospinal fluid: we recently had occasion to do this in two cases of pneumococcal meningitis, treated by intramuscular injections of penicillin only (2 million units two-hourly). The level found was never below 2 units/ml., which satisfied us that in this condition adequate diffusion into the cerebrospinal fluid takes place when the concentration in the blood is high, and that intrathecal injection should be unnecessary.

Perhaps more often, antibiotic assays are advisable to determine that the concentrations attained are not excessive. It seems not to be sufficiently understood that antibiotic excretion is a very sensitive index of renal function: when this is impaired, dangerously high blood levels are quickly attained. In some of the 20 cases of permanent vestibular damage described by Cawthorne & Ranger (1957), the dose of streptomycin responsible had been only a few grams, and the diagnoses in some of these cases, which include pyelonephritis and other renal affections, afford the explanation. Whenever a course of antibiotic treatment is necessary in a patient with known impairment of renal function, this factor must at least be carefully considered, and determinations of the actual concentration attained in the blood are a wise precaution. This situation perhaps most often arises in the treatment of subacute bacterial endocarditis, when some degree of embolic nephritis always exists. If renal infarction has been extensive, a condition usually betrayed by an episode of gross haematuria, it may be found advisable to reduce the dose of penicillin. In such a patient recently treated at St Bartholomew's Hospital, a blood level of 20 units/ml. four hours after a dose was found during treatment with oral penicillin V in a dose of 1 g. four-hourly, and when this dose was reduced to 0.5 g. four-hourly a further assay at the same time-interval showed

the presence of 16 units/ml. In another patient with bacterial endocarditis and severe renal involvement, receiving 3 mega units of sodium penicillin four-hourly, the blood concentration three-and-a-half hours after an injection was found to be 1,000 units/ml. Although penicillin is usually considered to be non-toxic, the attainment of such levels as this is probably inadvisable.

7. The Interests of the Community

We have considered hitherto only the interests of the individual patient, but the subject has a wider bearing. It is abundantly clear that the frequency of bacterial resistance to most antibiotics varies directly with the extent of their use in any given hospital: this is probably true of larger units of population. Thus every item of use of an antibiotic may detract by a very small fraction from its future usefulness. This is particularly true of such antibiotics as streptomycin, erythromycin and novobiocin, bacterial resistance to which can develop rapidly.

Various measures have been proposed to meet this difficulty, including restriction on use, rotational use, the isolation of certain classes of patient, and the use of combinations of antibiotics. We would make only one very general observation on it, namely, that it affords one of the strongest arguments against indiscriminate prescribing. So far as possible, an antibiotic should be prescribed only with the precise intentions regarding its effect which we have endeavoured here to define. Well-directed treatment limited to cases really requiring it may be expected to reduce total consumption and thus to extend the useful life of existing antibiotics.

PLATE I, FIGS. A, B

A. Plate culture of *Staphylococcus pyogenes*, incorporating tests of sensitivity to three antibiotics. The cup (hole) on the left was filled with a solution of penicillin (10 units/ml.). From the small diameter of the inhibition zone and the full size of the colonies bordering it, it can be seen that this strain forms penicillinase and is therefore resistant. It is sensitive to the other two antibiotics: these cups contain (upper right) streptomycin (250 μg./ml.) and (lower right) tetracycline (25 μg./ml.).

B. Primary culture of a sputum containing *Klebsiella pneumoniae* and *Haemophilus influenzae*. Cup on left contains penicillin (10 units/ml.) and, on right, tetracycline (25 μg./ml.). Both organisms are normally sensitive to tetracycline. The *Kl. pneumoniae* is exceptionally sensitive to penicillin, the *H. influenzae* (seen as small colonies within the zone of inhibition of the other organism) less so.

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PRINCIPLES OF THERAPEUTIC USE OF ANTIBIOTICS

(FIGS. A, B)

Lawrence P. Garrod & E. F. Scowen

(page 23)

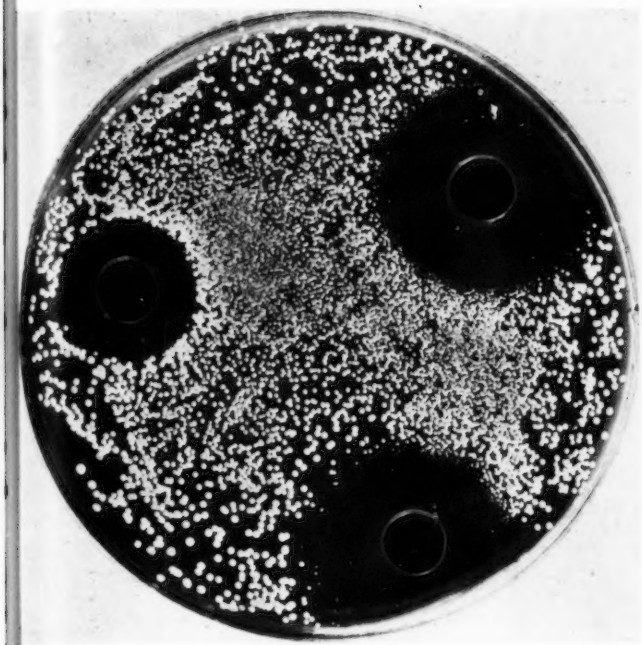


FIG. A

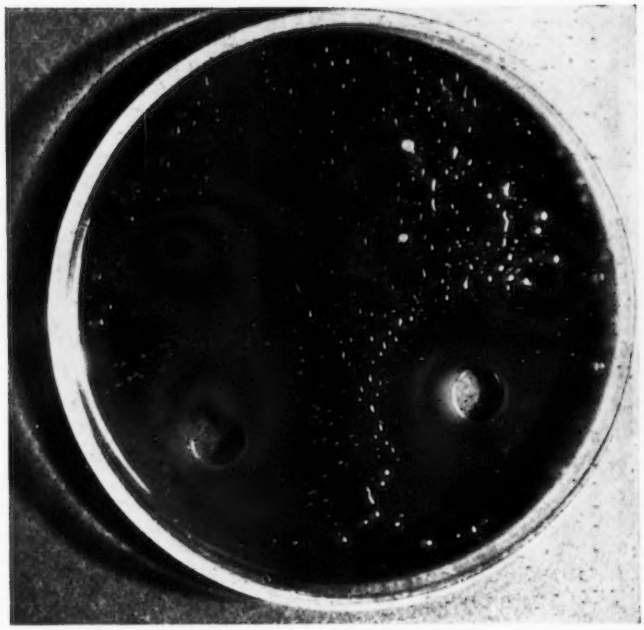


FIG. B

THE LABORATORY CONTROL OF ANTIBIOTIC THERAPY

(FIGS. C—H)

J. C. Gould

(page 29)

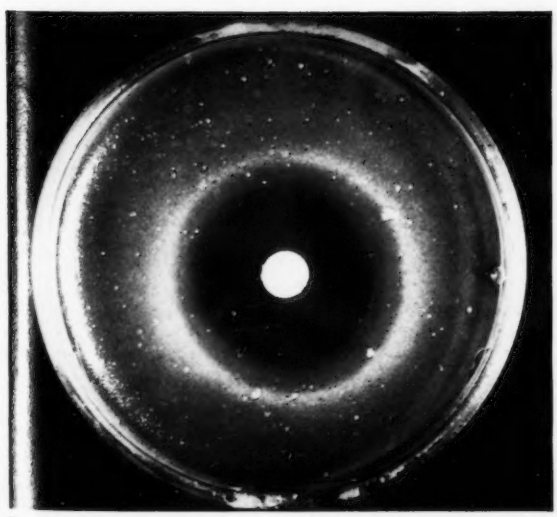


FIG. C

FIG. D

FIG. E

FIG. F

FIG. G

FIG. H

THE LABORATORY CONTROL OF ANTIBIOTIC THERAPY

J. C. GOULD B.Sc. M.B. Ch.B.

Department of Bacteriology
University of Edinburgh

1 Reasons for laboratory control
2 In-vitro determination of the sensitivity of micro-organisms to antibiotics
  a Serial-dilution tests
  b Diffusion tests
  c Quantitative interpretation of the disk-diffusion test
3 Sensitivity tests in practice
  a Primary sensitivity test
  b Subculture sensitivity tests
  c Assay of antibiotics
  d Antibiotic-resistant organisms
  e Testing of combinations of antibiotics
4 Significance of sensitivity tests
References

1. Reasons for Laboratory Control

In cases of infection, clinical diagnosis itself is not a dependable guide to the microbial flora present nor to their antibiotic-sensitivity, so that the first step towards the successful use of antibiotics is to establish a bacteriological diagnosis.

The clinician may direct specific antibiotic therapy merely by knowing the infecting organism, if it is a species whose antibiotic-resistant variants do not assume clinical importance (Garrod, 1955), and to this end a working knowledge of the antimicrobial spectra of each of the antibiotics in general use is of advantage. However, members of many species of micro-organism are not alike in their antibiotic-sensitivity and, as antibiotic-resistant cells have come to dominate many bacterial populations in hospitals and to a lesser extent outside of these, there is no reliable guide to the therapeutic use of antibiotics other than in-vitro sensitivity tests coupled to clinical experience. Therefore in most infections the sensitivity of the causative organism should be determined before specific therapy has begun and, if a specimen is available, and the clinician can afford a 24-hour delay, he should await the evidence of in-vitro tests. Within this time the most vital information concerning bacteriological diagnosis and antibiotic-sensitivity can be available, particularly so if helpful clinical details of the case are at hand. In this way the choice and dose of antibiotic can be guided by the findings in each individual case. Inadequate laboratory resources and inconveniently situated laboratories, or delay in the receipt of laboratory advice, discourage this or make it impossible.

The laboratory may assist further in bringing about a favourable outcome by measuring the efficiency of treatment. This may be carried out by detecting the presence of micro-organisms in body-fluids or exudates, and estimating any change in their antibiotic-sensitivity during therapy, and in selected cases by assaying the antibiotic content of body-fluids in order to ascertain whether or not an adequate concentration is being maintained. The time of cessation of treatment must next be decided; most frequently this is on clinical judgement, but frequently the bacteriologist may assist by confirming the eradication of the infecting organism. The withdrawal of an antibiotic may be expedient for other reasons, such as a change in the antibiotic-sensitivity of the causative organism, and this will require further advice as to the second choice of antibiotic.

PLATE I, FIGS. C, D (facing p. 28) AND PLATE II, FIGS. E—H

C. Disk-diffusion test. Agar plate, surface sown with overnight broth culture of *Staphylococcus aureus* and planted with a disk containing 1 μg. of penicillin. Several concentration zones can be seen. From within outwards these are: the zone of complete inhibition of growth; the zone of bacteriostasis where small colonies appear after longer incubation; the zone of bacteriolysis which appears after initial growth has taken place in the early hours of incubation, and may extend outwards; the zone of stimulation of growth where there is apparent enhanced growth; and finally the zone of normal growth.

D. Primary diffusion test. Primary culture of a urine containing *Escherichia coli* and *Streptococcus faecalis* on blood agar. The white circle on the right is a disk of Furadantin (10 mg.) to which both organisms are sensitive. The cups, reading clockwise, contained solutions of four other drugs:

Drug	Inhibition of	
	E. coli	*Str. faecalis*
Sulphafurazole (250 μg./ml.)	Moderately sensitive	Resistant
Streptomycin (250 μg./ml.)	Sensitive	Resistant
Tetracycline (250 μg./ml.)	Sensitive	Sensitive
Penicillin (100 units/ml.)	Resistant	Sensitive

E. Disk-diffusion subculture test. Agar plate, surface-sown with an overnight broth culture of *Staph. aureus* and planted with a disk containing 10 μg. of oxytetracycline. Within the zone of inhibition of growth of the majority of the bacterial population there are a large number of colonies, producing an almost confluent growth. These variants have a definite upper limit of sensitivity to the antibiotic, as shown by the well-defined inner ring of inhibition of growth.

F. Disk-diffusion subculture test. Agar plate, surface-sown with overnight broth culture of *Staph. aureus* and planted with disks containing 1 μg. of penicillin (left) and 10 μg. of streptomycin (right). There is no mutual effect of these antibiotics.

G. Disk-diffusion subculture test. Agar plate sown with a different organism from that in fig. F, and planted with disks containing 1 μg. of penicillin (left) and streptomycin (right). A small concentration of streptomycin mingling with a subinhibitory concentration of penicillin has produced complete inhibition of growth, demonstrating a synergistic effect.

H. Disk-diffusion subculture test. Agar plate, surface-sown with an overnight broth culture of *Staph. aureus* and planted with disks containing 1 μg. of penicillin (upper disk) and 25 μg. of chloramphenicol (lower disk). Colonies with a higher resistance to penicillin than the majority of the bacterial population are present and these are inhibited by low concentrations of chloramphenicol. This indicates an additive effect of the combination of the two antibiotics.

2. In-Vitro Determination of the Sensitivity of Micro-Organisms to Antibiotics

Laboratory control of antibiotic therapy therefore centres around tests for the in-vitro determination of the susceptibility of bacteria to antibiotics. These determinations are carried out by methods used in other forms of microbiological assay, the principles of which are shown in fig. 1. A concentration gradient of the antibiotic is prepared in a medium fully adequate to support the growth of the test organism, which is added uniformly to the mixture. Following incubation, growth occurs in the medium in concentrations of antibiotic below the inhibitory level and ceases where this level is reached, thus allowing assessment of the susceptibility of the micro-organism.

a. Serial-Dilution Tests

In practice, methods used to perform these tests include some form of serial-dilution test in which the concentration gradient is discontinuous; this is most frequently carried out as the serial-dilution tube test in which a series of tubes containing nutrient medium and varying amounts of antibiotic are inoculated with the organism under test (fig. 2). Following incubation, the tubes are examined for visible growth, which is compared with that in a control tube containing no antibiotic. The amount of antibiotic in the first tube showing diminished growth may be interpreted as the minimum inhibitory or bacteriostatic concentration, though frequently this interpretation is given to the first tube showing no visible growth. To determine the bactericidal concentration it is necessary to subculture from the tubes showing little or no growth, into medium free of inhibitory agent, and to ascertain the number of remaining viable organisms. The concentration of antibiotic in the first tube containing no surviving organisms is interpreted as the bactericidal concentration, that is, killing 100% of the bacterial population. This bactericidal concentration may be close to the bacteriostatic concentration, at other times many times greater, depending upon the antibiotic and the strain of micro-organism.

A similar technique may be followed using solid media in Petri dishes in place of fluid media in tubes. The antibiotic is incorporated in the medium in varying amounts, and the micro-organism is inoculated into the medium whilst molten, or sown on the surface after casting (May & Morley, 1952; Schatten & Parker, 1953).

The serial-dilution tube tests can be accurately performed and are easily interpreted in a quantitative manner. Their greatest drawback is the amount of time and material required to carry out a large number of tests, as may be required in routine clinical diagnostic work. A certain degree of skill is required to perform these tests, and unless care is taken, errors—especially pipetting errors—are likely to occur. Further, these tests require a pure culture of the strain of micro-organism for test and this necessitates initial isolation of the organism and subculture—a delay of 48 hours before a test result can be first obtained. Successful antibiotic therapy requires speed in the preliminary bacteriological investigations, and tests for antibiotic susceptibility must be considered as part of these. There is thus need for a simple, expeditious and reliable in-vitro technique for the determination of the susceptibility of bacteria to antibiotics. To fulfil these requirements some form of diffusion tests has been adopted by most laboratories.

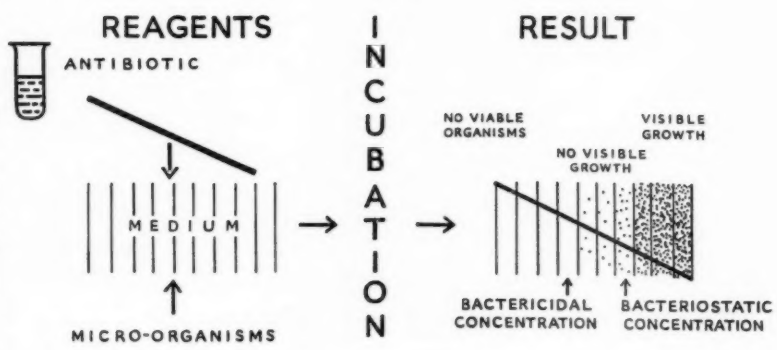
b. Diffusion Tests

In these the antibiotic is held in a reservoir from which it diffuses through the agar to form a diffusion gradient. The micro-organism in or on the agar is in contact with this gradient. The reservoir may be a hole or gutter cut out of the medium and filled with antibiotic solution or antibiotic mixed with agar (Fleming, 1929, 1942). Alternatively the antibiotic may be placed in a cylinder resting on the surface of the medium (Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey, 1941; Foster & Woodruff, 1943, 1944) or in absorbent paper disks (Pope, 1940; Morley, 1945; Fairbrother, Martyn & Parker, 1951; Gould & Bowie, 1952). Filter-paper is the most convenient form of absorbent paper likely to be uniform in quality so that pieces cut from the same grade of paper will absorb the same amount of fluid if they are of the same size.

Commercial disks for the tests are available, either impregnated or unimpregnated with antibiotics, and marked to indicate the nature of the drug. Some of these are of absorbent paper, others consist of a vehicle with antibiotic and a binding agent compressed into a tablet (Hoyt & Goolden, 1952). These have their disadvantages if the amount of antibiotic added to each disk or tablet varies (Branch, Starkey & Power, 1958; Sleigh, 1958), and prolonged storage under various conditions may lead to a falling off in potency of the material. The thicker tablets have not got the same area in contact with the medium as thin paper disks in relation to their volume; and release of antibiotic to the surrounding medium may be poor and inconsistent. Substances used as vehicles or binding agents in the tablets may not be biologically inert, and under test conditions may become antibacterial or interfere with the action of the antibiotic (Waterworth, 1958). For these reasons the standardization of commercial disks for such tests is desirable and certain steps in this direction have been taken (Greenberg, Fitzpatrick & Branch, 1957).

Filter-paper disks impregnated with antibiotic are easily and accurately prepared in the laboratory (Gould & Bowie,

FIG. 1. Principles of Antibiotic-Sensitivity Tests and Microbiological Assays



After Bowie (1953)

1952; Bowie & Gould, 1952) and can be stored and used in the wet state. The use of dried disks (Thomson, 1950) is more frequently described, but since a disk will become wet immediately on contact with agar this is no advantage. It has been claimed that drying prevents loss of potency on storage, but the difference is not significant (Gould & Bowie, 1952). Saturation of the disks may be carried out in bulk and, because of the uniformity of the paper fibre, each disk will contain the same amount of the antibiotic within sufficiently narrow limits (Gould and McInally, unpublished results). Saturation of the disks allows more uniform conditions for release of the antibiotic to the agar medium.

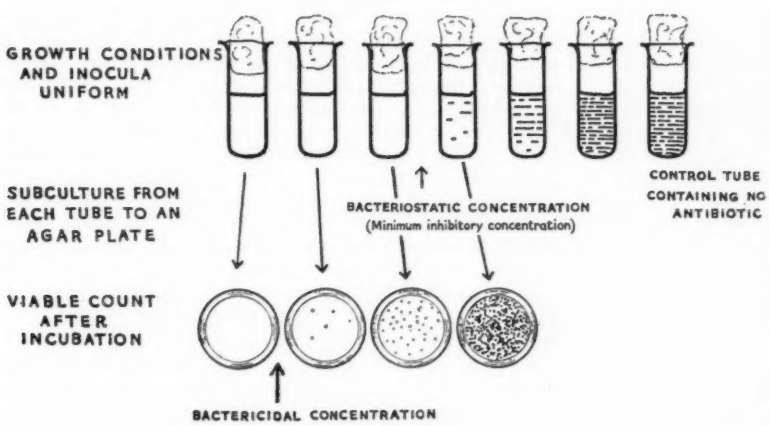
Diffusion of the antibiotic takes place continuously from the disk outwards through the surrounding agar so that the concentration gradient is continuously changing. The zones of inhibition of growth are circular and frequently complex (Pulvertaft, Greening & Haynes, 1947), showing the effect of different concentrations on the organisms during growth (Plate I, C); and their size depends upon factors which influence the diffusion of the antibiotic, such as pH (Topley, Lowbury & Hurst, 1951), depth, hydration and concentration of the agar (Schmidt & Moyer, 1944), and the nutrients and other substances dissolved in the gel (Loo, Skell, Thornberry, Ehrlich, McGuire, Savage & Sylvester, 1945), as well as the rate of growth of the test organism. However, since the rate of growth of the majority of the pathogens encountered in the clinical laboratory is similar and the other experimental conditions can be standardized, the disk test can give results of a high standard of reproducibility as well as a reasonable degree of accuracy. Replicate tests can be easily set up and the testing of a large number of different antibiotics undertaken on a single plate.

c. Quantitative Interpretation of the Disk-Diffusion Test

Quantitative tests have a wider application and therefore it is desirable to relate the zones of inhibition of growth to the amounts of antibiotic that are required for inhibition of growth. These are not the same as the amount of antibiotic in the disks, expressed as amounts per millilitre, as the concentration at a millimetre distant from the edge of a disk is several times greater than the actual amount in the disk and is minimal at the periphery of the medium. Thus it is quite erroneous to say that, because there is a zone of inhibition around a disk containing x µg. of antibiotic, the organism under test is susceptible to x µg./ml.

The diffusion of antibiotics in agar can be calculated on the assumption that known quantities of antibiotic diffuse regularly from a point source. Thus, the concentration of antibiotic present at a certain time, at a given distance from the centre of diffusion, may be calculated if the total amount of antibiotic used, the depth of the agar and the diffusion constant of the antibiotic in agar are known (Cooper & Woodman, 1946; Cooper & Gillespie, 1952; Cooper & Linton, 1952; Humphrey & Lightbown, 1952; Miyamura, 1953; Schlesinger, 1954). In practice this may be done for each antibiotic by plotting the zone of inhibition of growth of a standard organism against concentration of antibiotic in the source; graphs may be prepared for each antibiotic, and if necessary for a representative organism from each of the major groups likely to be tested in the laboratory (Gould & Bowie, 1952; Miyamura, 1953). More recently a novel method of measuring the amounts of antibiotic present in agar has been reported by Hoette & Struyck (1958), who punched out agar samples and estimated the amount of antibiotic actually present at various times of incubation.

FIG. 2. Serial-Dilution Tube Test


GROWTH CONDITIONS AND INOCULA UNIFORM
SUBCULTURE FROM EACH TUBE TO AN AGAR PLATE
VIABLE COUNT AFTER INCUBATION
BACTERIOSTATIC CONCENTRATION
(Minimum inhibitory concentration)
CONTROL TUBE CONTAINING NO ANTIBIOTIC
BACTERICIDAL CONCENTRATION

3. Sensitivity Tests in Practice

The exact identity of the micro-organisms present in pathological material is unnecessary for sensitivity determinations as long as the colonies are individually recognizable on the medium. An initial result can be obtained from primary cultures by placing antibiotic disks on suitably inoculated plates at the time of primary inoculation. The number of different media used and the atmospheric conditions of incubation must be varied so as to ensure growth of any species of micro-organism likely to be encountered.

a. Primary Sensitivity Test

The area of initial inoculation (reservoir) of the specimen is made so that it covers a sufficient area of the medium, and the material is spread as uniformly as possible. Antibiotic disks are applied within this area, their centres separated by two or more cm. (Plate I, D). After inoculation, the zone of inhibition of growth—if any—is examined and measured. In most cases this will give a satisfactory assessment, but it may be compared with the results of subculture tests at a later stage if desired. There will be a number of tests for which the inoculum is so sparse that the result cannot be assessed properly and in such cases recourse to a subculture test is necessary.

Where more than one species is present, it may not be possible to assess the sensitivity of individual species, because the growth of a relatively insensitive organism masks the growth of a more sensitive species. This may be unimportant as the sensitivity of the mixed flora is apparent, and sensitivity of individual species can be determined by tests on pure subcultures.

The primary test gives information usually adequate for clinical purposes, and no further sensitivity determinations

are required. It is quick and, in the great majority of cases, the clinician can have the results within 24 hours. The inoculum in this type of test is that present in the pathological material which—if the specimen is fresh—may be nearer to the actual number present in the body lesion. If multiple species are present, any reaction between them, for example in increasing or decreasing sensitivity to the antibiotic, will be apparent. In the primary test, the organisms are tested in a growth phase probably not too different from that in the body. In subculture tests the organisms may have become adapted to artificial culture and thus may have altered antibiotic susceptibility (McLeod & Mirick, 1942; O'Meara, McNally & Nelson, 1947). An additional advantage of the primary test is the occasional appearance within the zone of inhibition of numbers of resistant colonies belonging to another species of pathogen, otherwise unrecognized in the culture because of overgrowth. The presence of such an organism resistant to the antibiotic may greatly influence the outcome of therapy.

It is thus likely that a primary sensitivity test reflects more accurately the conditions which the antibiotics have to contend with in the body and so the results will be closer to those clinically obtained.

b. Subculture Sensitivity Tests

These may be carried out either by the disk or by the tube test as soon as the organism has been isolated.

Disk Tests

Individual colonies or a selection of colonies are subcultured in broth for 6–24 hours. These cultures are used undiluted, or diluted to the desired inoculum size, to inoculate the surface of agar plates. Antibiotic disks are then applied and zones of inhibition measured after 20 hours' incubation. Growth is usually confluent and the zone perfectly circular in shape so that it can be easily measured.

Tube Test

Subcultures in broth are used at the desired strength to inoculate a series of tubes containing antibiotic. These tests are particularly valuable when it is desired to estimate the susceptibility of organisms such as *Streptococcus viridans* isolated from blood in cases of subacute bacterial endocarditis and of *Brucella* in undulant fever, or when the simultaneous use of several antibiotics is contemplated and the maximum bactericidal effect of the combination has to be measured.

c. Assay of Antibiotics

Though rarely carried out now on patients undergoing treatment, the measurement of antibiotic in the blood or other body-fluid can give useful information. It is only by correlating the levels of antibiotic in the body with in-vitro sensitivity results that the significance of the sensitivity results can be assessed.

The methods used are similar to those for testing the sensitivity of infecting micro-organisms to an antibiotic, and that most frequently used is the serial-dilution tube technique (Rammelkamp, 1942; Grove & Randall, 1955).

d. Antibiotic-Resistant Organisms

One of the chief objects in carrying out in-vitro sensitivity tests is the detection of antibiotic-resistant members of bacterial population, since this is generally accepted as indicating potential resistance in the body, hence a poor therapeutic result. Failure to detect them may explain many instances where there is a discrepancy between the results of in-vitro sensitivity tests and results of antibiotic therapy.

Antibiotic-resistant variants almost always arise as a result of spontaneous mutation, and the frequency of these mutations usually varies from 1 in 10^6 to 1 in 10^{10}. The presence of such mutants in inocula of less than 10^6 cells is thus unlikely, but if they occur they will be more readily detected in an inoculum of 10^8 or more. Inocula of this size are more readily incorporated in tests using solid media than in those using fluid media, and the gradient plate technique of Szybalski (1952) is particularly convenient in this respect (Elek, Hilson & Jewell, 1953). The size of the inoculum frequently affects the end-point of these tests, and so a double inoculum test, measuring the susceptibility of a small and a large inoculum of the same species, has been advocated (Gilson & Parker, 1948). This test is of particular advantage when estimating the susceptibility of cultures of *Staphylococcus aureus* which may contain potential penicillinase producers.

The disk-diffusion test has the advantage that it gives an indication of the proportion of a bacterial population that is resistant to various concentrations of an antibiotic. In the tube test the presence of one viable resistant cell will result in growth. In the disk test it will only appear as a single colony within the zone of inhibition of the remainder of the population. If the proportion of resistant colonies is greater, the number of colonies appearing within the zones of inhibition will be greater and may have a definite upper limit of resistance, as shown by a well-defined inner ring of inhibition of growth (Plate II, E). The proof that these resistant variants are of the same species and strain as the main population depends on bacteriological identification of the colonies.

The presence of persistent viable organisms, within the zones of inhibition of growth around the disk on a plate, which have not grown into detectable colonies, may be shown by a modification of the Lederberg replica plate technique (Elek & Hilson, 1954). An impression sample of the sensitivity plate is taken with a felt pad and transferred to an uninoculated agar plate. Viable organisms transferred to this plate grow after incubation. This technique may be used to confirm a bactericidal (100%) concentration of antibiotic by the disk-diffusion technique.

e. Testing of Combinations of Antibiotics

Antibiotics in combination are being used with increasing frequency. This may be in an attempt to avoid the emergence of resistant mutants in the body during treatment, especially in the treatment of tuberculosis, or in the hope that an increased therapeutic effect can be obtained beyond the action of a single drug. The method may also allow the use of relatively toxic drugs in doses small enough to be tolerated by the host. The serial-dilution tube technique is most frequently used to demonstrate the activity of a mixture of antibiotics against a given micro-organism. Definite amounts of the components of the mixture can be added to the tubes in varying ratios, and the effect on growth of the micro-organism measured in detail. This has been done in a number of investigations by Jawetz (Jawetz, Gunnison, Coleman & Kempe, 1955) and in some cases a definite synergistic or antagonistic effect has been shown in vitro.

To cover a wide range of combinations of even two component drugs requires a large number of tubes and is a laborious procedure. An alternative method is that of Martin,

Sureau & Chabbert (1952), in which tubes of broth containing antibiotics singly and in combinations are inoculated with the organism and plated after incubation to determine whether the effect has been merely bacteriostatic or actually bactericidal. Complete sterility in this subculture offers promise of successful therapeutic effect, particularly in bacterial endocarditis. Some information on the combined effects of two or more antibiotics in vitro can be obtained by using the disk technique after the manner of Lamanna & Shapiro (1943). Disks are placed with their centres at a suitable distance so that the respective antibiotics will diffuse into one another to give a continuous range of relative concentrations in the early hours of incubation when definitive zones of inhibition are produced. Ideally this may be done after subculture tests when the sensitivity of the micro-organism to drugs A and B has been determined independently, and the position of disks containing A and B can be predetermined to produce the desired concentration mixtures; for example, A and B may be placed x mm. apart, where $x = a + b$, a being the radius of zone of inhibition with A, and b the radius of the zone obtained with B. After incubation the two zones of inhibition will make contact and there will be a thin wedge of growth on either side of the point of contact of the zones (Plate II, F). When there is appreciable additive synergistic inhibitory effect (Kolmer, 1945) of individually subinhibitory concentrations of A and B, the growth in the area of the wedges will be inhibited and can be seen without difficulty (Plate II, G), or resistant growth within the zone of inhibition of one drug may be inhibited by the diffusion of relatively small quantities of the other (Plate II, H). The concentration of the individual components producing these inhibitory effects may be inferred from the standard graphs in the usual way by measuring the distances from the individual disks to the area of inhibition. Mutual interference (antagonism) between antibiotics can be shown in the same way, though in our experience it is rarely observed.

4. Significance of Sensitivity Tests

The value of all laboratory sensitivity tests must be the correlation between their results and those in the patient receiving specific therapy. There is general agreement that these tests are of value to the clinician but there is a paucity of factual information, with the result that we are as yet at an elementary stage in correlating the clinical administration of antibiotics with in-vitro sensitivity determinations designed to give some idea of the amount of antibiotic required. Thus, although the in-vitro bacteriostatic and bactericidal concentrations of antibiotics for any bacterial species can be ascertained, these are not used for deciding the dosage, and reliance is placed on standard "dosage schedules" of antibiotics.

The problem can be solved by comparison of the results of in-vitro tests and of therapy with individual antibiotics, by bacteriologists and clinicians. There are relatively few reports on investigations of this sort but those that have been made are encouraging in that there appears to be a reasonably high degree of correlation, indicating that the advice is helpful (Giertz & Gullbring, 1951; Broom, Martineau & Young, 1953; Rodger, Branch, Power, Starkey, Gregory, Murray & Harrop, 1956). Clinicians involved in the treatment of tuberculosis have generally had a closer liaison with the laboratory than those treating other infections, and many attach great significance to the results of in-vitro sensitivity tests (Crofton & Stewart, 1957).

It is often forgotten that the levels of penicillin maintained in the blood in cases of serious staphylococcal and streptococcal infections in the early days of antibiotic therapy were of the same order as the in-vitro inhibitory level of these organisms, and in most the clinical results were dramatically favourable (Mackie, 1946). More recently the work of Eagle, Fleischman & Levy (1953) has shown that the effective levels in vivo are of the same order of magnitude as those effective in vitro and, in an experiment carried out in relation to urinary tract infection (Gould, Bowie & Cameron, 1953), a level of antibiotic in urine, calculated from the results of in-vitro tests and confirmed by assay, sterilized the urine in 14 of 17 cases.

Discrepancies and inconsistencies between laboratory and clinical results do occur. Clinical success may follow a laboratory report that the organism is resistant. This contretemps occurs most frequently in the case of *Staph. aureus* infections treated with penicillin and is undoubtedly due to the peculiarity of this organism in producing penicillinase when in the resistant form, so that the result of an in-vitro test is very dependent on the size of the inoculum. The primary sensitivity test may be of particular advantage in dealing with this problem (Bowie, 1953). Clinical failure may occur when a report of in-vitro tests indicates susceptibility of the infecting organism. This also occurs frequently in the case of *Staph. aureus* infections, and also with urinary tract infections due to *Escherichia coli* and other coliform bacilli. Here a lack of response is likely to be due to failure to correct an underlying cause of the infection, but acquired bacterial resistance may also be responsible and it is advisable to examine the urine at frequent intervals during treatment so that the drug may be changed if necessary.

Any micro-organism within living tissue and accessible to an antibiotic is likely to be more sensitive than in any in-vitro test, for in these tests the organisms are usually more numerous per unit volume than is ever likely to be the case in the tissue. In vitro the organism may also be temporarily in a resting phase (Chain & Duthie, 1945), in which state it is more resistant to inhibition; also the natural antibacterial mechanism of the host increases the activity of the drug (Parker & Luse, 1948; Eagle, 1948; Eagle & Musselman, 1949), which varies in degree from patient to patient. The micro-organism, on the other hand, may be relatively inaccessible in the tissues even although the amount of antibiotic circulating is sufficient to inhibit the organism. Only clinical trial can show with certainty that the organism infecting a particular patient is sensitive or resistant to the antibiotic in the particular dose exhibited. Thus, all things considered, the arbitrary dividing line between sensitive and resistant in in-vitro tests may be too high, and the tendency for the bacteriologist is to underestimate the inhibitory power of the antibiotic, particularly if he knows little of the clinical details of the case (Tunevall & Ericsson, 1954).

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THE PHARMACOLOGY OF ANTIBIOTICS

J. M. ROBSON M.D. D.Sc. F.R.S.Ed.

Department of Pharmacology
Guy's Hospital Medical School, London

G. A. H. BUTTLE O.B.E. M.A. M.R.C.S. L.R.C.P.

Department of Pharmacology
School of Pharmacy, University of London

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The useful antibiotics are derived from either streptomyces or penicillia. The use of bacteria as a source of antibiotics is severely limited by the fact that all antibiotic substances so far derived from them are polypeptides which give rise to varying degrees of kidney damage. Polymyxin B and E, and to a more limited extent bacitracin, are the only antibiotics of bacterial origin which can usefully be employed and even these have a very limited sphere. The antibiotics produced from streptomyces, on the other hand, with the single exception of neomycin, and to a much lesser extent streptomycin, do not give rise to this difficulty. (See Jawetz, 1956.)

Recently a great deal of work has been done to produce chemical modifications of the antibiotics. These modifications have often produced substances giving longer persistence in the body and sometimes greater concentrations in particular tissues.

1. Penicillins

Shortly after the isolation of penicillin and the elucidation of its chemical structure (see review by Clarke, Johnson & Robinson, 1949), it was realized that there are a number of penicillins, all with similar chemical structure, but with rather different pharmacological properties. Of those originally isolated, benzylpenicillin was considered to be the most suitable and is now extensively used. It suffers from the disadvantages that it is comparatively ineffective when given orally and that its period of action, when given by injection, is comparatively short. Further work on penicillin has been done with the following main aims: (i) to obtain preparations with more prolonged effects; (ii) to obtain preparations effective orally; and (iii) to obtain forms of penicillin active against organisms resistant to benzylpenicillin. The first two objects have been largely achieved, but the third one still eludes investigation. Progress has been achieved by producing new forms of penicillin and by producing pharmaceutical preparations, essentially of benzylpenicillin, which modify its pharmacological properties. Biosynthetic methods have been largely used successfully in the production of new forms of penicillin, but chemical methods are also at present being used.

There are two international standard preparations of penicillin, i.e., the sodium salt of benzylpenicillin and the free acid of phenoxymethylpenicillin. Preparations of penicillin can thus be assayed biologically, but the British Pharmacopoeia also gives chemical methods for the determination of benzylpenicillin and of total penicillins.

Benzylpenicillin (Penicillin G)

Benzylpenicillin is usually administered by intramuscular injection, as a sterile solution of the sodium or potassium salt, in water to which a suitable buffering agent may be added. It is rapidly absorbed from the site of injection and distributed throughout the body. The maximum plasma concentration is reached about half an hour after injection and then falls rapidly to reach very low values after a few hours; the actual duration does of course depend on the dose given. Serum levels following administration of various doses have been worked out by Tucker & Eagle (1948). The maintenance of a satisfactory penicillin level involves about four intramuscular injections over the 24 hours, and Tucker & Eagle (1948) give tables which show the frequency at which a given dose should be injected or the dosage of penicillin which should be given at stated intervals in order to maintain a given concentration of penicillin in the plasma. Ungar (1950) has shown that, following the intramuscular injection of penicillin, the drug is present in tissue exudates much longer than in the blood, but the experiments of Eagle & Musselman (1948) suggest that this may not be true for an area of acute inflammation without tissue breakdown. Penicillin is therapeutically active when combined with the albumin fraction of plasma proteins, though it is not known whether this combination is chemical or physical. Much of the penicillin is excreted in the urine, either unchanged, or as inactive metabolites. About 80% of the penicillin excreted by the kidney passes through the tubules, and substances which inhibit tubular activity, e.g., probenecid, will thereby tend to raise the penicillin level in the blood and tissues. Such a method may be of value when very high levels of penicillin have to be attained, as in the treatment of certain cases of bacterial endocarditis.

Penicillin does not pass very well through the blood-brain barrier unless the meninges are acutely inflamed, though with high doses effective concentrations may be produced in the cerebrospinal fluid (c.s.f.) (Redfearn, Elithorn, Till & Ibbott, 1949). It can be given intrathecally, though the dose should be kept low, otherwise there may be severe meningeal reaction. An intrathecal dose will maintain a chemotherapeutic level in the c.s.f. for about 24 hours. The drug diffuses readily from maternal to foetal blood. It passes well into ascitic fluid and to some extent into pleural, pericardial and synovial fluids, as well as into the bile (Hirsh & Putnam, 1958). The distribution of this and other important antibiotics in body-fluids after administration by the usual route for obtaining a systemic effect is summarized in Table I.

Benzylpenicillin is poorly absorbed from the gastrointestinal tract and some of it is also destroyed there. Though

TABLE I. Distribution of Antibiotics in Body-Fluids after Usual Route of Administration

(Adapted from Table 5, James (1957), by permission of the publishers)

Agent	Usual route	Urinary excretion	Cerebro-spinal fluid	Serous cavities	Bile
Benzylpenicillin	Intra-muscular	Rapid	Unsatis-factory	× ¼*	× 5*
Tetracyclines	Oral	Slow	Unsatis-factory	× ¼–½*	× 10*
Chloram-phenicol	Oral	Rapid	× ½*	Free diffusion	× ½*
Streptomycin	Intra-muscular	Rapid	Unsatis-factory	× ¼–½*	× ¼–½*
Erythromycin	Oral	Rapid	0	Unsatis-factory	×8–64*
Polymyxin	Intra-muscular	Slow	0	0	0
Novobiocin	Oral	Slow	0	Readily detected	× 16*

* Relative to blood level

a variety of methods have been used to improve the absorption of benzylpenicillin, such as administration with antacids, it is now generally accepted that phenoxymethylpenicillin is a more satisfactory oral preparation.

Until recently penicillin was rather extensively used for local application, e.g., to the skin or the eye, and the 1953 British Pharmacopoeia contains a number of preparations suitable for local application. Allergic effects are, however, common with this method of administration, which is best avoided. The preparations for local use have in fact been withdrawn from the 1958 Pharmacopoeia.

Procaine Penicillin

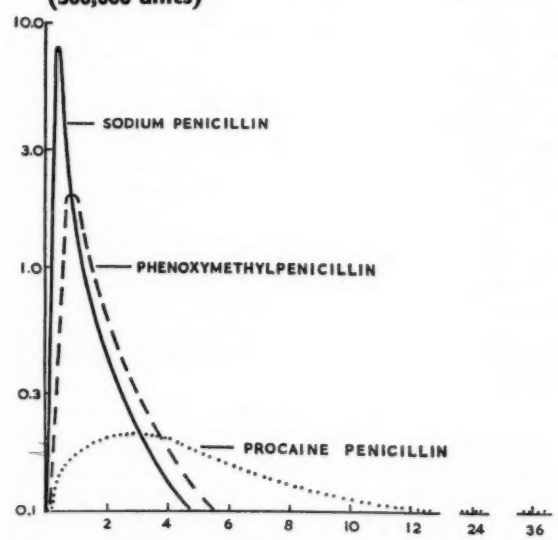
A disadvantage of benzylpenicillin is that its period of action is short, so that repeated injections are necessary in order to maintain a suitable level in the tissues. A variety of methods have been used to extend the period of action of penicillin, e.g., suspension in oil. The commonest preparation now used is procaine penicillin which is prepared by the interaction of procaine hydrochloride and benzylpenicillin. It is suspended in water with a suitable dispensing agent and, usually, a buffering agent. Such a preparation will not produce as high a blood level as an equivalent dose of benzylpenicillin and the maximum level will be attained more slowly, but a chemotherapeutic concentration will be maintained for about 12–24 hours (Herrell, Nichols & Heilman, 1947). It is, therefore, very suitable for treatment in all but severe infections. A dose of benzylpenicillin can be incorporated into the preparation of procaine penicillin in order to attain more rapidly a suitable level in the tissues. A water-repellent agent, such as aluminium monostearate, is sometimes added to the suspension of procaine penicillin in oil, and this will still further increase the period of action to several days, though the level attained is correspondingly decreased (Thomas, Lyons, Romansky, Rein & Kitchen, 1948). This preparation is not much used.

Phenoxymethylpenicillin (Penicillin V)

This is usually dispensed in tablets and is the most satisfactory form of penicillin for oral administration; 125 mg. are approximately equivalent to 200,000 units of penicillin. Following administration of 240 mg., the peak level is reached after about one hour (0.5–8.0 units/ml.), but the concentration is low after four hours, so that even with this high dose, repeated administration is necessary to maintain an adequate concentration (Colquhoun, Scorer, Sandler & Wilson, 1957). The superiority of phenoxymethylpenicillin as compared with benzylpenicillin, when both are given orally, has been clearly demonstrated by a number of investigators (e.g., Heatley, 1956; Griffith, 1958). Heatley found that 20–25% of an oral dose was excreted in the urine, whether the drug was taken while fasting or immediately after a meal. The curve of the blood concentration produced by an oral dose of this form of penicillin is compared with that produced by an injection of sodium or procaine penicillin G in fig. 1.

Benzathine penicillin (*NN'*-dibenzylethylenediamine penicillin) is a preparation only very sparingly soluble in water, and has been included in the 1958 British Pharmacopoeia for both intramuscular injection and oral administration. On intramuscular injection, the blood level gradually rises and is maintained for a long period, in some cases for over three weeks, that is, it gives serum penicillin levels of greater

FIG. 1. Blood Levels of Penicillin following Administration of Different Penicillin Preparations (300,000 units)



Abscissae: time (hr.)
Ordinates: units/ml. of penicillin (logarithmic scale)

——— sodium penicillin G, intramuscularly (Garrod, 1950)
— — — — phenoxymethylpenicillin, orally (Griffith, 1958)
..... procaine penicillin G, intramuscularly (Whittlesey & Hewitt, 1948)

24 and 36 hours after the injection of procaine penicillin the mean blood level was approximately 0.05 units/ml.

duration than with any other form of depot penicillin. The injection is, however, frequently rather painful (Fletcher & Knappet, 1953). It may be combined with procaine penicillin which gives higher initial concentrations and does not affect the slow absorption of the main preparation (Welch, Randall & Hendricks, 1953). The effectiveness of benzathine penicillin is illustrated by the work of Perry & Gillespie (1954) who gave monthly intramuscular injections to rheumatic children and almost completely prevented new infections. The findings of Foltz & Schimmel (1953) on blood levels and urinary excretion do not suggest that in adults benzathine penicillin is particularly well absorbed when given orally, though in children this preparation appears to be more satisfactory by this route (Cathie & MacFarland, 1953).

Allylmercaptomethylpenicillin (penicillin O) has a distribution in the human subject similar to that of benzylpenicillin (Adair, Woodin & Bunn, 1951). It was introduced for use in patients hypersensitive to benzylpenicillin, but there can be a good deal of cross-sensitization between the two drugs (Siegals, 1951).

6-*Aminopenicillanic acid.* Recently Batchelor, Doyle, Nayler & Rolinson (1959) have described the production of this from *Penicillium notatum.* They used a medium free of added side-chain precursors and produced this hygroscopic substance from the culture media. The substance is less active than benzylpenicillin, but it is thought that large-scale production may lead to interesting compounds which can be more readily prepared chemically from the free amine than by adding various substances containing acyl groups to the culture filtrate. This work is still in progress.

2. Streptomycin and Neomycin

In 1916 Waksman & Curtis isolated the actinomycete *Streptomyces griseus* from the soil, and 28 years later, following the progress in bacterial chemotherapy initiated by the discovery of sulphonamides and penicillin, he obtained streptomycin from the organism. Later, vitamin B_{12} was isolated from the same culture medium. Dihydrostreptomycin was made in 1946 by Peck, Hoffhine & Folkers by the catalytic hydrogenation of streptomycin, and in 1949 Waksman & Lechevalier isolated neomycin from another actinomycete, *Streptomyces fradiae.* There are international standards of streptomycin, dihydrostreptomycin and neomycin, against which preparations of these drugs are assayed biologically. The British Pharmacopoeia recognizes the sulphates of these three antibiotics, but gives no direction about the use of neomycin.

Streptomycin and Dihydrostreptomycin

These drugs behave in the same way in the body, though there are differences in their toxicity. The data given about the administration, distribution and clearance of streptomycin also apply to dihydrostreptomycin. Streptomycin is very poorly absorbed from the intestine though, unlike penicillin, there is little inactivation in the gut, and the drug can thus very effectively be used for a local action in the intestine. For a systemic effect it is usually given by intramuscular injection. The concentrations attained, when related to the body-weight, indicate a distribution in the extracellular fluids, and there is indeed good evidence that it penetrates poorly into cells (Mackaness, 1952). Following intramuscular injection in man, the maximum blood concentration is reached in 1–2 hours and falls to very low values in 6–7 hours (Marshall, 1948). There is evidence that the concentration of streptomycin produced in the blood is proportional to the dose per kg. of body-weight, within a definite range of dosage (Boxer, Jelinek, Tompsett, DuBois & Edison, 1948). Much of the drug is excreted in the urine, but this varies in different subjects, and in some cases a considerable amount of the dose given is not accounted for by urinary excretion; the excretion appears to be essentially by glomerular filtration. The drug is concentrated and excreted by the liver into the bile and can pass through the placental barrier into the foetal circulation (Zintel, Flippin, Nichols, Wiley & Rhoads, 1945), though the concentration in the cord blood and amniotic fluid is generally less than half that found in the maternal blood (Woltz & Wiley, 1945). When streptomycin is given intramuscularly in the usual doses, very little diffuses into the c.s.f. In acute meningitis, however, much greater diffusion occurs, though the concentration is still less than half that found in the blood serum (Adcock & Hettig, 1946). Streptomycin can safely be given intrathecally (not more than 100 mg. in each dose) and effective levels are maintained for up to 30 hours. Small amounts of the antibiotic are excreted in milk, saliva and sweat, and negligible amounts enter the red blood cells and thick wall abscesses; the drug penetrates well into necrotic tuberculous lesions in the lungs of man (Canetti & Grumbach, 1956).

Neomycin

This is a very stable substance and is said to be less liable to produce allergic effects than streptomycin, particularly when applied locally. Being poorly absorbed, it is therefore used for oral administration to produce an effect in the intestine, and for local application to various parts of the body. On the other hand, it is liable to be more toxic than streptomycin when given systemically and should therefore be used by this route only with caution, since it is quite liable to produce toxic effects on the kidney and on the eighth nerve, particularly involving hearing. Intramuscular injection should not be continued for any length of time. Neomycin is sometimes effective against bacteria resistant to streptomycin.

3. Other Antituberculosis Antibiotics

Viomycin is produced by the growth of *Streptomyces puniceus.* It is an antituberculosis agent effective against streptomycin-resistant organisms, though less active than streptomycin and more toxic, and hence of limited clinical usefulness. Viomycin is usually given by intramuscular injection and is largely excreted by the kidneys. The diffusion into the c.s.f. is poor (Werner, Adams & DuBois, 1951). Very little of the drug is absorbed when given orally.

Cycloserine is a substance of known chemical structure, produced by the growth of *Streptomyces orchidaceus.* It has also been synthesized. It is effective against a variety of organisms, including *Mycobacterium tuberculosis,* but its activity is rather low and toxicity high. It is readily absorbed when given orally and excreted rather slowly in the urine of man and the monkey, though more rapidly in lower species (Welch, Putnam & Randall, 1955; Conzelman, 1956). Significant levels are produced in the c.s.f., foetal blood and amniotic fluid (Anderson, Worth, Welles, Harris & Chen, 1956).

Kanamycin is produced by *Streptomyces kanamyceticus* and has been found to be effective against a number of

organisms, including *Myco. tuberculosis*. It is not absorbed when given orally and therefore is administered by intramuscular injection, a single dose producing a chemotherapeutic level for about eight hours (Bunn & Baltch, 1958; Steenken, Montalbine & Thurston, 1959).

4. Chloramphenicol

Chloramphenicol was isolated in 1947 by Burkholder and his colleagues at Yale University (Ehrlich, Bartz, Smith, Joslyn & Burkholder, 1947); it was produced by a strain of streptomyces isolated from a soil sample collected in Venezuela. It is a crystalline substance and was first called chloromycetin, and its chemical identity was soon determined as D-(−)-*threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1:3-propanediol. It is now prepared synthetically and the synthetic material is found to be as effective as the natural product. A number of chemical modifications of chloramphenicol have been prepared (Hahn, Hayes, Weisseman, Hopps & Smadell, 1956), but none of them is as active as chloramphenicol itself.

Method of administration. Chloramphenicol is well absorbed when given orally and also when given rectally (El Borolossy, 1953). It is used in capsules of 250 mg. for oral administration, and can also be used intravenously and intramuscularly, but the oral absorption is so rapidly effective that these other routes are seldom required. Chloramphenicol palmitate is sometimes used for oral administration as this palmitate ester does not possess the bitter taste of chloramphenicol itself. The palmitate is rapidly hydrolysed in the intestine, and the toxicity and activity are similar to those of the parent substance.

Rate of absorption and concentration in blood, urine, and c.s.f. Chloramphenicol is rapidly absorbed, and a dose of 1 g. orally in man will rapidly give a serum level of 20 mg./l.; this falls to about half its original level in six hours (Burnell & Kirby, 1951). These authors also investigated the levels of the drug after intravenous administration and found them to be similar to those obtained by the oral route.

Chloramphenicol is very easily diffusible and therefore probably reaches a higher concentration in the tissue than many other antibiotics. Its beneficial action in typhoid fever may well be dependent on this diffusibility, for the tetracycline antibiotics are as active as chloramphenicol on *Salmonella typhosa* in vitro, but they are not effective in the infected animal or in the patient. The diffusibility of the substance also makes it very suitable for topical use in ophthalmic work, and for local applications of all kinds.

Werner & McDermott (1954) devised a method for growing *Salm. typhosa* on agar plates implanted into the peritoneal cavity of cats. They found that chloramphenicol and chlortetracycline were effective in the peritoneal fluid in similar concentrations in delaying the growth of the organism; but it was necessary to give two to four times the dose of chlortetracycline to the cat to get a concentration in the peritoneal fluid similar to that found with chloramphenicol.

Chloramphenicol is found unchanged in the serum after administration by mouth or parenterally. About 60% of it probably occurs as a bound form in the serum. It diffuses readily from the blood; the highest concentrations are found in the kidney and liver, and the lowest in the brain and spinal fluid. It enters the spinal fluid with relative ease even in the absence of meningeal inflammation, and after a single dose of 1–2 g. in adults it appears in the c.s.f. in 1–3 hours, a peak concentration being obtained in 4 or 5 hours; this is about 25–50% of the concentration found in the blood, the difference being presumably due to the binding of the drug with the protein in the plasma. It penetrates readily into pleural and ascitic fluids and crosses the placental barrier with ease; no toxic effects have been discovered in infants.

Chemical assays indicate that 75–90% of orally administered chloramphenicol is excreted in the urine within 24 hours of administration; this is in a form in which the nitro group is intact, yet microbiological investigations reveal in the urine only 10% of the dose administered (Ley, Smadell & Crocker, 1948; Ross, Bischoff, Preisser & Orr, 1949). This suggests that chloramphenicol is inactivated in the body, and hydrolytic products and glucuronic acid conjugates have been found by chemical examination of the urine (Smith & Worrel, 1950); these latter forms are without significant antibiotic activity. In spite of the presence of these inactive products the concentration of unchanged chloramphenicol is high, exceeding 200 μg./ml. after a dose of 1.5 g. orally. The unaltered chloramphenicol is probably excreted by the glomeruli, and probenecid has no effect on the amount found in the urine (Boger, Matteucci & Beatty, 1951).

5. Tetracyclines

There are three antibiotics in this group, i.e., chlortetracycline (aureomycin), oxytetracycline (terramycin), and tetracycline itself; in addition to these, a number of other substances have been prepared synthetically from tetracycline.

Of all these, chlortetracycline was the first to be produced from *Streptomyces aureofaciens* by Duggar (1948) of the Lederle Laboratories, Pearl River, New York, while oxytetracycline was obtained later from *Streptomyces rimosus* by a research team at the laboratories of Pfizer & Co. Tetracycline itself was produced from a streptomyces isolated in soil from Texas by the Hayden Chemical Corporation. Tetracycline is now usually prepared from chlortetracycline by chemical means.

Method of administration. The tetracycline antibiotics are usually given in capsules or tablets by the oral route. They can, however, be employed intravenously or intramuscularly. Injections of tetracycline and oxytetracycline, containing procaine, are available for intramuscular injection; as both produce some pain at the site of injection, the procaine is used to reduce this discomfort (Montmorency, 1954).

Absorption from oral administration is not so rapid as with chloramphenicol. The antibiotics are detectable in the serum within an hour of administration. The maximal level is reached in 2–4 hours, and there is a fall to a very low level in 24 hours. Chlortetracycline is more rapidly destroyed in neutral and alkaline solution than the other two and after oral administration the blood level falls to zero in 24 hours, but the rate of this fall depends very much on the dose administered. About 70% of the chlortetracycline in the plasma is bound to the plasma proteins (most of it to the albumin fraction), and this may help to explain the persistence of this unstable antibiotic in the blood (Sirota & Saltzman, 1950). In dogs the amount found in the gastrointestinal tract four hours after administration has a linear relation to the dose from 5 g./kg. to 25 g./kg.—about 60% of the dose given could be recovered in this way (Gray, Hill, Winne & Cunningham, 1953).

The tetracycline antibiotics diffuse poorly into the c.s.f.,

and the concentration here is of the order of one-tenth of that present in the blood. Tetracycline itself finds its way into this fluid in a slightly higher concentration than do the other two compounds. The concentrations found in the c.s.f. are higher and more dependable if the antibiotics are administered by the intravenous route than if they are given by mouth, and hence this route is to be preferred during the first few days of treatment in a case of meningitis. Tetracyclines are found in the pleural fluids and in ascitic fluid; in the latter the concentrations are less predictable. The antibiotics are found in the bile and the saliva and diffuse fairly readily across the placental barrier. Large quantities are excreted in the bile; some of this is reabsorbed, which accounts for the persistence of the antibiotic in the blood.

Tetracycline or its metabolic derivatives appear to be concentrated in bone and remain there for up to 10 weeks after administration. Milch, Rall & Tobie (1957) have found that the fluorescence seen in most tissues after tetracycline administration disappears in 6 hours; in bone, however, this fluorescence persists for 10 weeks and is most marked where new bone is being formed. Anderson, Ferguson & Braude (1959) have shown that bacteriostatic activity remains, in the bone showing this fluorescence, up to 96 hours after the drug has been given.

Excretion. After the administration of 0.5 g. of tetracycline, 20–60% is found in the urine during the ensuing 24 hours. Significant amounts of tetracycline are found in the faeces; if 2 g. of tetracycline a day are given by the oral route there are concentrations of up to 2.2 mg./g. in the wet stool, and, even when 0.5 g. is given intravenously, small amounts of up to 18 μg./g. are found in the stools (Maynard, Andriola & Prigot, 1953). With oxytetracycline the larger the oral dose given, the smaller is the proportion absorbed, and so the concentration in the faeces rises sharply as the dose is increased (Herrell, Heilman, Wellman & Bartholomew, 1950). This may result in greater disturbance of bowel function, with inhibition of the growth of normal intestinal bacteria and an increased risk of proliferation of *Candida albicans*.

Chemical modifications of tetracycline. Several efforts have been made to prepare modifications of the substance that will persist in the body for a longer time than tetracycline itself. Kaplen, Albright & Buckwalter (1957) prepared a phosphate complex of tetracycline, and Pulaski & Isokane (1957) have shown that this complex is more rapidly absorbed than tetracycline and gives a 50% higher blood level with the same dose. Welch, Wright & Staffa (1958) report that oral administration of tetracycline with an equal quantity of glucosamine hydrochloride produces a quicker absorption of the antibiotic by the oral route and a higher level in the blood. The same differences occur with oxytetracycline; this is confirmed by Bunn & Cronk (1958). Kunin, Jones & Finland (1958) also confirmed the higher levels obtained from phosphate or glucosamine complexes and state, moreover, that a mixture of equal parts of tetracycline and of citric acid may give even better results.

Kunin & Finland (1958) have investigated the compound 6-demethylchlortetracycline. First described by McCormick, Sjolander, Hirsch, Jensen & Doerschuk (1957), this compound gives much higher and better-sustained levels in the blood than tetracycline itself.

6. Erythromycin

Erythromycin is produced by *Streptomyces erythreus*, which was originally isolated from a sample of soil collected in the Philippines. It is a useful drug to have in reserve when other antibiotics are ineffective. It is standardized biologically in comparison with an international standard.

Erythromycin is usually given orally and is reasonably well absorbed by this route, though high gastric acidity tends to destroy the drug and prevent absorption. It can be given intravenously as the glucoheptonate, and intravenously or intramuscularly as the lactobionate. Maple, O'Leary & Kirby (1953) have shown that erythromycin given intravenously disappears rapidly from the blood stream to reach a low value in three hours. Erythromycin is also applied locally to the skin or eye, as an ointment containing 5–10 mg./g.

Following oral administration, the plasma concentration reaches a maximum at 3–4 hours, and appreciable amounts are still present at 8 hours. Some of the drug is excreted in the urine to give adequate chemotherapeutic concentrations. Diffusion into the c.s.f. is irregular (Grigsby, Johnson & Simmons, 1953), but the drug passes through the placental barrier. It is concentrated in the liver and appears in the bile; moderate amounts appear in the faeces (Heilman, Herrell, Wellman & Geraci, 1952). The distribution of erythromycin in various tissues in rats has been studied by Lee, Anderson & Chen (1953), who found that the drug was concentrated in various organs and that the tissue levels decreased less rapidly than the serum level. Further investigations, using radioactive erythromycin, have also been reported by these workers; they showed that erythromycin and its metabolites can enter various cellular components of the liver; they also studied the renal excretory product of the drug (Lee, Anderson & Chen, 1956).

Various esters of erythromycin have been made and it has been shown, in preliminary studies, that propionyl erythromycin produces a higher and more sustained level in the blood than erythromycin base (Griffith, Stephens, Wolfe, Boniece & Lee, 1958).

Spiramycin, oleandomycin and carbomycin. The pharmacological behaviour of these substances is similar to that of erythromycin except that carbomycin is less well absorbed: this was a major reason for the abandonment of its therapeutic use.

7. Novobiocin

Novobiocin base is not absorbed, and the calcium or sodium salt is therefore used therapeutically; the former is more stable and is employed when a liquid preparation is wanted. The sodium salt is rapidly absorbed, a peak level in the blood being reached in 2–3 hours; the level remains high for 8 hours and there is a small quantity left in the serum after 24 hours. The antibiotic diffuses into pleural and ascitic fluid but does not reach the c.s.f. if the meninges are not inflamed. Novobiocin is concentrated in the liver and bile and excreted in the faeces with small quantities in the urine (Furesz, 1958). In dogs the urinary excretion is very slow and it seems likely that the maintenance of high concentrations in the blood is due to the slow renal excretion and also perhaps to binding to serum proteins (Taylor, Miller & Vander Brook, 1956).

8. Vancomycin

Vancomycin is produced by *Streptomyces orientalis* and its main action is against gram-positive organisms, the chief indication being infections due to *Staphylococcus aureus* resistant to other antibiotics. The drug must be given intra-

venously to produce any action other than in the intestine. After intravenous administration vancomycin is rapidly excreted in high concentrations in the urine, and it should therefore be given in several daily doses, e.g., six hourly. It is not concentrated in the bile and is detectable in pleural, pericardial, synovial and ascitic fluids. It does not diffuse into the cerebrospinal fluid. After oral administration most of the drug is excreted in the faeces, and only slight amounts can be detected in the blood or urine (Geraci, Heilman, Nichols, Welman & Ross, 1956).

Ristocetin is a substance closely similar to vancomycin and behaves in a similar way (Fairbrother & Williams, 1958).

9. Antifungal Antibiotics

Nystatin

Nystatin is poorly absorbed by mouth and almost completely excreted in the faeces. There is no evidence for absorption from mucous membranes. It is used orally to prevent intestinal infection with *C. albicans* when broad-spectrum antibiotics are employed and is also used locally for treatment of infection of mucous membranes with *C. albicans*.

Amphotericin B

Amphotericin has been used by the oral route, and a very small blood level of 0.04–0.50 μg./ml. has been found. This level is sufficient to produce some fungistatic effect against *C. albicans*, but intravenous administration of the antibiotic is more satisfactory and 0.6 mg./kg. intravenously gives a blood level of 2–3 μg./ml.; this is maintained at fungistatic levels for long periods. The levels found in the c.s.f. are very low (Louria, 1958).

Two patients with systemic mycoses with meningeal involvement were treated with intravenous amphotericin at two-day intervals, using 0.75 mg./kg. It was found that the toxicity was low and that a satisfactory fungistatic level could be maintained in the serum (Vogel & Croutcher, 1958).

Griseofulvin

Griseofulvin is the metabolic product of *Penicillium griseofulvum*. When administered orally to guinea-pigs, it has been found effective in infection with *Microsporum canis* or *Trichophyton mentagrophytes* (Gentles, 1958); the antibiotic is found in the hair (Gentles, Barnes & Fantes, 1959). Lauder & O'Sullivan (1958) have also shown that it is effective against *Trichophyton verrucosum* in calves. The dose in both cases was 60 mg./kg. daily. The oral toxicity of the antibiotic in rats and mice is very low. Further work on the treatment of human infections is now being carried out (Williams, Marten & Sarkany, 1958; *Lancet*, 1958).

10. Polypeptide Antibiotics

Bacitracin

Bacitracin is a polypeptide derived from *Bacillus licheniformis* of the *Bacillus subtilis* family. It is not absorbed by the oral route, but can be used systemically by intramuscular injection. There is an international standard, and preparations should not contain less than 50 units/mg. (British Pharmacopoeia, 1958).

Like all bacterial polypeptides it may produce swelling of the kidney tubules; the smaller doses produce a temporary albuminuria with some increase in cells, and sometimes casts. Doses of 3,000 units/kg./day for 37 days caused albuminuria and kidney damage in monkeys (Welch, 1954). Larger doses may cause a rise of blood urea. The urinary output is usually increased and a fall in the output should preclude further administration of the drug.

Polymyxin

Polymyxin B is a polypeptide from *Bacillus polymyxa*. There is an international standard for polymyxin B, and preparations should not contain less than 6,000 units/mg. It is given locally and parenterally. It does not penetrate into the c.s.f., and intrathecal injections are necessary in meningitis. Other forms of polymyxin, A, C and D, have been abandoned owing to toxicity to the kidneys; with polymyxin B and E, however, this effect is not troublesome, although transient albuminuria may occur after parenteral use of larger doses of the antibiotic. Owing to the possible toxicity the antibiotic is used for *Pseudomonas aeruginosa* infections only when no other agent is available (Jawetz, 1956; Swift & Bushby, 1953).

Tyrothricin

Tyrothricin, which comprises two antibiotics, gramicidin and tyrocidine, is obtained from *Bacillus brevis*. It is not absorbed orally and is dangerous to use parenterally owing to its nephrotoxic effect: hence local application to skin or mucous membranes is its only field of usefulness.

11. Antibiotics and Malignant Disease

Actinomycin (Waksman, 1954) inhibits the growth of certain animal tumours and has been used in Hodgkin's disease (DiPaolo, Moore & Niedbala, 1957).

Azaserine (*O*-diazoacetyl-L-serine), as well as being effective against certain micro-organisms, inhibits the growth of some experimental tumours (Stock, Reilly, Buckley, Clarke & Rhoads, 1954). Pharmacological data are given by Sternberg & Philips (1957).

DON (6-diazo-5-oxo-L-norleucine) is effective against a number of bacteria (Ehrlich, Coffey, Fisher, Hillegas, Kohberger, Machamer, Rightsel & Roegner, 1956). It is also effective in certain animal tumours and has been tried in patients (Magill, Myers, Reilly, Putnam, Magill, Sykes, Escher, Karnofsky & Burchenal, 1957).

* * *

In conclusion it may be said that there have been rapid developments in this subject. The discovery of penicillin has been a stimulus to the development of a new and constantly expanding field in therapeutics. The more recent advances, however, have on the whole merely provided better antibiotics for the same infections, whether by greater antibacterial activity or by improved pharmacological behaviour: no progress has been made in the discovery of drugs effective against the smaller viruses. Antibiotics have been found which are highly active in inhibiting the division of mammalian cells, and this development may eventually contribute towards the chemotherapy of malignant disease.

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THE RATIONALE AND MANAGEMENT OF COMBINED THERAPY

B. W. LACEY B.Sc. M.D.

Westminster Hospital School of Medicine, London

A Rationale of combined therapy
1 Therapy of the individual patient
a Increasing the intensity of antimicrobial action
b Increasing the chances of effective therapy
c Prolonging the period for effective therapy
d Reducing the chances of superinfection
2 Maintenance of therapeutic value of antibiotics in the environment
3 Reduction of cost

B Management of combined therapy
1 Tactics
2 Short-term strategy
3 Long-term strategy
References

A recent leading article (*Lancet*, 1957) on "Antibiotic combinations" ended with the following: "Meanwhile, we hope that every doctor who may have occasion to prescribe an antibiotic combination will carefully examine and assess the evidence before he does so". In fact, however, there is almost no evidence to assess because the present practice of chemotherapy has been developed largely by trial and error and under the influence of local fashion and publications of persons with a financial or emotional interest in a particular drug or mode of therapy. Wiser advice would therefore seem to be: "We hope that every practising doctor will construct and refresh at intervals his own set of principles, and that he will base these on critical and authoritative reviews and his own carefully considered experience."

Among the many recent reviews of combined therapy, the following appear particularly valuable: Garrod (1953), Elek (1956), Dowling (1957), Jawetz (1958), Knothe & Witt (1958), Pryles (1958), Tompsett (1958) and Weinstein (1958). In all of these the authors have taken the view that the use of two drugs can be justified only in certain limited circumstances. This critical unanimity has already had the healthy effect of damping down all enthusiasm for combinations. It has also, and probably more importantly, dammed back, for the moment at least, the potential torrents of factory-made mixtures of antibiotics, and mixtures of an antibiotic with almost any other therapeutic substance, which threatened to reduce to chaos a field already much confused. In the present paper, therefore, the problems of combined therapy have been considered from the two complementary aspects of rationale and management. References to original work have been reduced to a minimum, partly because most of importance are to be found in the reviews mentioned above and partly because in this sphere, perhaps more than in most, the number of references affords no measure of the worth of the author's opinions.

A. RATIONALE OF COMBINED THERAPY

In general one can recognize three primary reasons for giving two or more chemotherapeutic agents at the same time: to improve the therapy of the individual patient, to maintain the value of such drugs in the same environment, or to reduce cost. Only the first reason has been discussed in most recent reviews; but, though at present this is by far the most important consideration, it may not always be so, particularly in the chemotherapy of cancer. It seems opportune therefore to consider all three.

1. Therapy of the Individual Patient

Two drugs may achieve a therapeutic or "tactical" synergy in one or any combination of four ways: by increasing the over-all intensity of antimicrobial action, increasing the chances of success in "blind therapy", prolonging the period of effective therapy or reducing the chances of superinfection by either the patient's own or the hospital flora. The term synergism is often reserved for the first of these ways. In this paper, however, it will be used to include any effect, either in vitro or in vivo, which either could not be achieved at all by one drug alone in any dosage or concentration, or could not be produced by one drug alone so often or so quickly, or without more intoxication, expense or labour.

a. Increasing the Intensity of Antimicrobial Action

Though much is now known of the possible modes of action of many antimicrobial agents, the experimenter's inability to reproduce in vitro the "open system" conditions which exist in vivo, and the many unpredictable factors influencing absorption, distribution, breakdown and excretion, make it impossible in most instances to do more than hazard a guess at the precise action and effect of giving any chemotherapeutic substance. With two or more drugs, even less predictable factors become important—such as their relative rates and degrees of penetration to different sites within the zone of inflammation or into parasitized cells, and the relative concentrations, rates of accumulation and effectiveness of their antagonists. It follows that almost all practice of combined therapy under this heading has been developed by trial and error and that only in retrospect has any rationale been defined. The numerous ways in which two substances may exert a synergistic antimicrobial action have recently been reviewed (Lacey, 1958). But though an analysis of this kind may be of long-term strategic interest, it is of little help to current therapy. In retrospect it has become clear that the most important distinction to make at present is between combined therapies which lead to rapid sterilization of lesions and those which do not.

i. More Rapid or Complete Stasis

In most infections the natural defences of the body are sufficiently effective to eliminate the micro-organisms, or at least continuously suppress their multiplication. In these, chemotherapy need—and usually does—only reduce the rate of multiplication of the organisms (ideally to zero) for the outcome to be assured, and as a rule one bacteriostatic drug is almost certainly as effective for this purpose as two. Two drugs nevertheless probably achieve a more certain stasis or killing, and on these grounds appear indicated (but not of course necessarily justified) under the following conditions:

i. with relatively insensitive organisms such as, for example, with *Pseudomonas pyocyanea* infections of the urinary tract treated with a combination of polymyxin and a tetracycline. Here any advantage possessed by the two drugs over one probably depends on a suppression of the outgrowth of resistant mutants rather than on a "cytotoxic" synergy in which the two drugs can be shown in vitro to be synergistic against a uniform population of organisms;

ii. with two or more organisms of different sensitivity as, for example, with the combination of penicillin and streptomycin in treatment of peritonitis following perforation of the lower bowel, where streptomycin inhibits the coliforms and penicillin inhibits the cocci, *Clostridia* and at least some of the fusiforms (*Bacteroides*);

iii. with relatively inaccessible organisms such as, for example, with the combination of streptomycin and a tetracycline in undulant fever. *Brucella suis* organisms may be about 25,000 times more sensitive to streptomycin when naked than when ingested in leucocytes (Shaffer, Kucera & Spink, 1953) and it thus seems likely that the empirically discovered advantage of the two drugs in brucellosis is bound up in some way with different penetrating powers of the two drugs for the parenchymatous cells of the liver and the reticulo-endothelial cells in which the brucellae multiply. A slightly different situation obtains when, as in trypanosomiasis, an organism penetrates to a place completely inaccessible to a drug which is otherwise an effective agent. Thus in late trypanosomiasis it is necessary to give tryparsamide for its ability to penetrate the blood-brain barrier and, after reduction to the trivalent form, inhibit trypanosomes in the central nervous system (CNS), as well as suramin for its highly effective action outside the CNS;

iv. on the comparatively rare occasions when in-vitro tests show the two drugs to have a markedly synergistic static action, that is to say when the concentration of each drug in combination needed to inhibit growth is much less (often 4–10 times less) than that of the same drug alone;

v. when the microbial population is likely or almost certain to include highly resistant mutants which would outgrow the sensitive population within hours or days. This is usually the case with drugs such as streptomycin, erythromycin and novobiocin to which many organisms show one-step mutations to a high degree of resistance—even 1,000-fold or more—and bacteria with a short generation time, such as members of the *Proteus*, *Pseudomonas*, *Escherichia*, *Aerobacter*, *Salmonella*, *Haemophilus* and *Staphylococcus* genera;

vi. when the usual single drug of election is contra-indicated for any reason (such as intractable sensitivity, on epidemiological grounds or through difficulties in administration to an unconscious patient); and when one or more of conditions (i)–(v) above also exist.

ii. More Certain or Rapid Sterilization

In some infections a deficiency of the defences prevents or markedly delays elimination of the micro-organisms. If the deficiency is a temporary or remediable one, such as the lack of curative antibodies in scrub typhus treated at the onset of disease, the cure is obvious. But after massive irradiation, during steroid therapy, or with a permanent and irremediable defect, the chemotherapy must lead directly to sterilization of the lesions if it is to have lasting value. Very occasionally a permanent lack of antibody, as in hypogammaglobulinaemia, may be found responsible; but defective phagocytosis almost certainly underlies most permanent deficiencies of defence. Both microphages and macrophages may naturally lack the capacity to ingest the microbe, as in actinomycosis, cryptococcosis, moniliasis and trypanosomiasis; or there may be insufficient leucocytes, or the phagocytes may be unable to reach the microbes either because of avascularity, as in subacute endocarditis, or because obstructed by old pus (as in some empyemata and infections of bone and soft tissue) or by other dead tissue or a foreign body.

In all these situations it seems justifiable at present to consider using a combination of two or more drugs which, either from previous experience is known or can reasonably be presumed to be more effective than one, or has been shown in vitro to have a rapid synergistic killing action on the isolated organism. In retrospect, then, the truth of what Garrod (Cates, Christie & Garrod, 1951), Jawetz (Jawetz & Gunnison, 1952) and Elek (Elek, Hilson & Jewell, 1953) have emphasized for many years has become apparent to all: that when the natural defences are deficient, the laboratory findings become more important than usual and can best indicate the drug combination of election from among a bewildering maze of possibilities. What method the laboratory should best use for this purpose is not known, though on logical grounds the latest method of Chabbert (1957) would seem the one of choice, with the method of Elek & Hilson (1954) a close second (and a good deal simpler).

b. Increasing the Chances of Effective Therapy

In serious, undiagnosed infections a combination of agents of complementary ranges of activity is more likely to be effective than any single substance. Blind therapy of this kind then seems justifiable provided that: (i) all reasonable steps to establish a diagnosis, including collection of specimens for laboratory examination, have been taken beforehand; (ii) substances are chosen which will least reduce the chances of eventually establishing a definitive diagnosis; (iii) the patient's progress is reviewed in the light of clinical and laboratory findings as soon as possible and not later than the fourth day of treatment. Thus, for example, with meningitis of unknown origin it is most desirable to take a specimen of cerebrospinal fluid before giving any therapy, since organisms such as the meningococcus may be irrecoverable within 30 minutes of swallowing a dose of a sulphonamide. In the same disease, in order not to jeopardize the subsequent isolation of a tubercle bacillus, it is equally desirable to avoid giving streptomycin blindly.

c. Prolonging the Period for Effective Therapy

In most infections the natural and acquired defence mechanisms become fully developed within 12 days. *A priori*, therefore, infections which progress or fail to regress after this time are manifestations of deficient resistance and, in retrospect, it is not surprising that they do in general require more potent and more prolonged therapy. Hence, in chronic infections, the use of two or more drugs is sometimes justifiable to avoid one or both of two obstacles to prolonged therapy: the outgrowth of resistant mutants, and intoxication.

In tuberculosis the use of two agents is too well known to need comment but, though their practical value in preventing the emergence of resistant mutants is well established, it is not clear whether they achieve this by inhibiting the outgrowth of mutants already present at the beginning of treatment or by inhibiting growth of mutants arising subsequently from

partially inhibited organisms. The former seems the more probable mechanism, especially in view of recent evidence that streptomycin and isoniazid can have a direct synergistic antimycobacterial action (see Meadow & Knox, 1956).

The use of dihydrostreptomycin and streptomycin in combination to reduce ototoxicity also appeared rational after Heck & Hinshaw (1953) had reported finding no damage to the eighth nerve in 42 persons with tuberculosis who were given the mixture. But the use of the two drugs fell to a very low level when Wier, Storey, Curry & Schless (1956) showed how unfavourably results with the mixture, in their hands, compared with previously published results with streptomycin alone. To sum up, however, though it is reasonably certain that dihydrostreptomycin is the more toxic to auditory function, and streptomycin is the more toxic to vestibular function, the relative toxicity of the mixture is not evident. No properly controlled simultaneous comparison of all three regimes has yet been made on unselected cases with strictly comparable histories and microbial sensitivities and with a patently unbiased clinical and audiometric assessment. Even if the mixture were then to prove more toxic to auditory function than streptomycin alone, a lower vestibular toxicity might still at times justify its use. Though auditory loss is very serious for a physician or a musician, it is probably less so than vestibular dysfunction for a surgeon, an air-pilot or a ballet dancer.

d. Reducing the Chances of Superinfection

One has only to suck penicillin lozenges for a week to prove how much one's well-being normally depends on the penicillin-sensitive flora of the mouth. There seems little doubt that the gram-negative organisms of the faeces, and especially perhaps *Escherichia coli*, have a similar protective action for the bowel mucosa and that their suppression inevitably leads to further change in the flora and a risk of a pathogen's becoming predominant. At worst the new flora may cause a dangerous or lethal enteritis as well as constituting a new reservoir of infection for other patients, and if this is established during therapy it is almost certainly drug-resistant. But even if established after chemotherapy, it may still be drug-resistant.

One must conclude therefore that it is clearly incumbent on anyone who prescribes chemotherapy to consider and minimize its effect on the bowel flora. In therapy this means at times either employing a single drug such as a sulphonamide, ristocetin, or perhaps erythromycin, with a narrow enough or feeble enough activity not to lead to this complication; or giving by mouth a second agent such as neomycin, nystatin, chlorquinaldol, bacitracin, ristocetin or vancomycin, to suppress the upgrowth of the pathogen. After chemotherapy it means facilitating when necessary the re-establishment of an innocuous flora. At present in hospital this is probably best done by giving orally, for three or four days after withdrawing the other therapy, a narrow-spectrum antistaphylococcal agent, such as bacitracin, vancomycin or ristocetin, and large numbers of lactobacilli or *E. coli*, or any other organism which may prove more effective.

2. Maintenance of Therapeutic Value of Antibiotics in the Environment

At present the problem of using drugs in combination for this purpose, in what may be called a "strategic synergy", arises only with tuberculosis and staphylococcal disease. But the solutions in the two diseases differ greatly. In the first, the strategic objective is to prevent the appearance in the sputum, or other exudate, of drug-resistant tubercle bacilli in sufficient numbers to be infective; and the means of achieving this coincide exactly with those for the therapeutic objective, already discussed, of prolonging the period of effective therapy.

In contrast, in staphylococcal infection there is little convincing evidence that resistant mutants have arisen by mutation from the original sensitive strain.[1] Indeed, in the case of penicillin it is now generally accepted that penicillin-resistance virtually never arises in this way, and in the case of novobiocin there is at least an indication that resistant organisms may often be superinfectors rather than new mutants (see, for example, Pearson, Somberg, Rosenthal, Lepper, Jackson & Dowling, 1956; Nichols & Finland, 1957). It follows logically (i) that the important reservoirs of drug-resistant staphylococci are surfaces and wounds colonized or infected by drug-resistant organisms; (ii) that both the administration and withdrawal of chemotherapy or chemoprophylaxis of any kind should be planned with due regard to the dangers of creating new staphylococcal reservoirs; (iii) that, wherever possible, drugs or combinations of drugs should be chosen which least predispose to the establishment of such reservoirs. With surfaces such as burnt skin, or the alimentary tract, this is probably most readily achieved by using singly or in combination substances such as neomycin, bacitracin or (on burns) chlorhexidine, which are of negligible value in systemic therapy and to which the staphylococcus develops little resistance. With infected wounds (whatever the infecting organism) the problem is more complex and it would seem necessary to weigh up (a) the possible clinical advantage of using a combination of two effective drugs in order to prevent the outgrowth of resistant mutants, against (b) the risk to other patients, from superinfection of the site by a staphylococcus resistant to both drugs. At present in hospital (b) clearly often outweighs (a), whereas in a patient's home (a) almost always outweighs (b).

3. Reduction of Cost

The cost to the community of any course of therapy depends on many factors whose relative importance varies greatly in different circumstances. Thus in hospital the length of the course is often the dominant factor, whereas at home the cost of the drugs or of their administration often equals or exceeds the duration of therapy in importance. In Britain we are fortunate in that the choice of regimes is seldom dictated by questions of material cost. Nevertheless many opportunities arise for economy without loss of efficiency and some of these justify the use of two drugs in place of one. Thus penicillin and a sulphonamide will often achieve as much as a tetracycline by itself, if not more; yet the respective costs of a day's therapy are 4s. 2d. and 9s. Similarly the cost per day of giving streptomycin and sulphadimidine is about 2s. 8d., whereas those of nitrofurantoin alone and neomycin alone are about 7s. 2d. and £1 respectively.

Even if the cost per day of two were more than that of one, the use of two drugs could still in theory be of advantage economically, in terms of material cost as well as the cost of occupying a bed, and justifiable clinically, if the two reduced the period of therapy needed to achieve the same

[1] See Waisbren, B. A. & Strelitzer, C. L. (1959) *Amer. J. med. Sci.* **238**, 202

result. Perhaps the nearest approach to this situation occurs in brucellosis. In this disease Killough (1957) found a strong indication that the only demonstrable effect of combining streptomycin with a tetracycline was to reduce the period of therapy needed for cure from 6–8 weeks to 3–4 weeks. If this proves to be the rule, then each case of brucellosis will have to be assessed before therapy, and the dangers of streptomycin set against the expected economy of time and money.

There is also evidence from several recent records that subacute endocarditis caused by penicillin-sensitive viridans streptococci (for which a dose of 2–4 million units of penicillin a day for 4–6 weeks is usually prescribed) may be permanently cured by a 14-day course of penicillin and streptomycin. However, in the latest of these (Tompsett, Robbins & Berntsen, 1958) the results of a course of 6 million units of penicillin and 2 g. of streptomycin a day for 10–18 days were not clearly better than those of penicillin alone for 28 days, and could hardly be held to outweigh the unpleasantness and potential toxicity of some 30 g. of streptomycin. Moreover, as Tompsett (1958) himself points out, the fever which continues during healing, and after withdrawing the penicillin and streptomycin, greatly obscures the clinical recognition of persistent infection.

Finally, in general practice it must at times be much less costly, though equivalent clinically, to give two drugs orally rather than one that demands a daily visit by a visiting nurse.

B. MANAGEMENT OF COMBINED THERAPY

The management of combined therapy, like that of warfare or any other complex, fluid and long-lasting problem, is conveniently considered from three aspects: firstly in terms of immediate tactics when confronted with a patient, then as an exercise in short-term strategy, which is more or less a mixture of clinical preparedness, common sense and opportunist research on the individual patient, and thirdly as a long-term strategic problem which calls for planned research in both clinic and laboratory.

1. Tactics

First choose the best combination; then make the best use of it.

Selecting the Best Combination

1. Exclude from first consideration all fixed-dose mixtures. None is suitable for routine use, because the timing and doses suitable for one organism in one person are seldom those of choice for another person or another organism. They are more costly, confuse terminology and aggravate the difficulties of assessment and determining the cause of side-effects. But the most important reason for avoiding them is that their use strongly tends to inhibit thought and the development of a rationale and hence the only safeguard of sanity in this field.

2. Make a short list of drugs or drug combinations which appear likely to be effective because they are: (i) shown in vitro to have a synergistic lethal action on the isolated organism; or (ii) shown in vitro to have separate static actions on the isolated organism; or (iii) known to be effective as a rule in vivo, either separately or together, against the species of organism known or presumed to be present; or (iv) known to be effective in vivo or in vitro against the organism in the presumptive source of infection.

3. From this list choose combinations known in general to have a synergistic killing action and avoid those known at times to have an antagonistic action. At all times, but especially with subacute bacterial endocarditis, avoid combinations of penicillin with chloramphenicol or any tetracycline, or any other predominantly static drug, unless there is a clear indication from laboratory or clinical results that the combination is not antagonistic. Many combinations have been shown to be antagonistic under specified conditions either in vivo or in vitro but, with the exception of those with penicillin mentioned above, it seems most improbable that they are ever significantly so in therapy. Certainly the risk of a slight antagonism of this kind should not be taken to contra-indicate a combination which, in other respects, such as by delaying the outgrowth of resistant mutants, can be presumed usefully synergistic.

4. Do not plan to keep a drug of election in reserve. The best time to use almost any antimicrobial agent (pyrazinamide is a notable exception) is at the height of fever and during rapid multiplication of the organism. This particularly applies to isoniazid in tuberculosis.

5. In preference, choose components with the most killing action, narrowest spectrum, to which the organism shows a multi-step pattern of resistance and which in bacteriologically undiagnosed cases appear least likely to add to the difficulties of establishing a definitive diagnosis. Of course, components with least toxicity, lowest cost and easiest administration should also be chosen.

6. Always consider combining drugs to which organisms show a single-step pattern of resistance, such as erythromycin, novobiocin and streptomycin, with another agent in order to delay or prevent the outgrowth of highly resistant mutants.

7. Bear in mind the potential disadvantages of combined therapy: increased cost; increased risk of sensitization; increased difficulty of establishing a diagnosis and a decreased incentive to do so; increased toxicity (especially for the kidney, with two nephrotoxic drugs; for the eighth nerve, with two neurotoxic drugs; and for the liver, with two drugs such as terramycin and pyrazinamide); and increased chances of removing enough gram-negative organisms from the bowel to court the risk of an enteritis or allow the establishment of another reservoir of hospital staphylococci.

Using the Combination to Best Advantage

8. In general, prescribe each component of a mixture in full dosage. Cytotoxic synergy is usually of a low order even when demonstrable and, except perhaps with penicillin and streptomycin in enterococcal endocarditis, should not be relied on.

9. Start treatment as early as possible, both for obvious reasons and to minimize the size of the microbial population and hence the chances that resistant mutants might be present from the start.

10. As with single-drug therapy, always consider the possible necessity for surgery, treatment with steroids, antitoxins, antisera or vaccines, for altering the inflammatory environment to achieve the maximum antimicrobial action (such as making the urine alkaline before giving streptomycin) or for increasing absorption or decreasing storage, breakdown or excretion.

11. Also as with single-drug therapy, take samples for laboratory examination before starting therapy and review every patient within the first four days of treatment in the light of clinical progress and laboratory findings.

2. Short-Term Strategy

1. Use no combination without a specific reason but particularly none with a component (such as at present erythromycin or novobiocin) whose value in any patient is liable to be inversely proportional to the extent of its recent use in other patients in the same environment.

2. Keep yourself informed of the drug sensitivity of the bacteria in the presumptive sources of infection by contact with maternity departments, by keeping a book noting ward infections, or by other means.

3. Seek a cause for individual failures. Were the drugs and doses prescribed actually given and absorbed? Was the organism finally isolated still sensitive in vitro to the drugs used? Was there a lack of any specific or non-specific defence mechanism?

4. View information from drug houses critically, whether published overtly in a circular or as an apparently unbiased scientific paper.

5. Do not uncritically accept for use any combination based only on a laboratory evaluation or on an uncontrolled or inadequately controlled trial in man. Do not, however, dismiss experiments in the test-tube or with mice as of little or no value because not immediately applicable to man. The number of factors influencing the outcome of therapy in man is far too large to allow their relative importance and interaction to be explored directly. Indeed, as Box (1958) points out, only laboratory experiments permit the investigator to gain a general idea of the shape of the response surfaces, and hence the basic information for designing an experiment with human beings likely to be least dangerous and most profitable of time and money.

3. Long-Term Strategy

Among the many unanswerable questions in combined therapy the following seem of special importance and to call for deliberate research.

1. *Correlation of in-vivo and in-vitro findings.* What in-vitro test of sensitivity or synergy best predicts in any disease the results of combined therapy? It seems certain that evidence of rapid synergistic killing of bacteria is usually much more significant than that of synergistic stasis. But when, if at all, are tests for stasis helpful? And which among the numerous tests for killing is best for any given circumstance? How does the optimum in-vitro method vary with species or strain of organism and with sex, age, constitution and site of disease?

2. *Relative value of different regimes.* What in any given disease is the value, in prophylaxis or treatment, of no therapy, or of therapy with one, two or three drugs? Properly controlled therapeutic trials are never easy to organize and seldom cheap to run, yet their results so often upset orthodox ideas, and prove of lasting value, that they should clearly be made more often than at present. Most persons probably think only of hospitals in connexion with therapeutic trials. Yet there appear to be exciting possibilities for the latter in general practice. As Box (1958) suggests, "evolutionary operation", aimed at achieving by successive approximations the best prophylaxis and therapy available at any time, could well be conducted in the course of normal practice. It demands only three conditions, all more or less available for the asking: an efficient and co-operative service of diagnostic aids; a central agency to collate, integrate and plan; and a willingness to revisit, review and report.

3. *The causes of failure.* Can failure to respond be associated with an odd pharmacological fate of the combination in the body, an odd genetic constitution, or to some other abnormality, from which the failure might have been predicted beforehand?

Besides these questions needing clinical research for an answer there is, of course, still unlimited scope for laboratory research. At one extreme is the most fundamental and intellectually satisfying approach, whose ultimate therapeutic goal is the logical synthesis of new agents and the logical prediction of highly synergistic combinations. At the other is the tedious, albeit potentially more immediately productive one, of random screening of compounds and living things for antimicrobial activity. Between these there is a vast field for semi-empirical search among compounds either known already to have biological activity, or closely related physico-chemically to these, or similarly related to agents of proved chemotherapeutic value. A non-inhibitory substance may be a synergist in one or more of many ways (see Lacey, 1958) and it seems certain that an extensive exploration of these groups would reveal many useful synergists and also much information of general biological interest. Some studies along these lines have indeed already been reported (Sallman & Streitfeld, 1959; Scherr & Bechtle, 1959).

In sum, it appears clear that all three aspects of the management of combined therapy should be the concern of all who are responsible for the prescription of chemotherapy, and of all in the laboratory who handle infected material.

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PREVENTIVE USE OF ANTIBIOTICS IN MEDICINE

E. G. L. BYWATERS M.B. F.R.C.P.

Professor of Rheumatology
University of London

Honorary Director
Medical Research Council Rheumatism Research Unit
Canadian Red Cross Memorial Hospital, Taplow
Maidenhead, Berkshire

1 Circumstances warranting prophylaxis
2 Hazards and drawbacks of prophylactic antibiotics
3 Practical use of antibiotics in prevention of rheumatic fever and rheumatic heart disease
References

Resistance to infectious disease depends primarily upon good general health and the adequate development of those specific immunological defences which derive from previous experience of the infecting agent. While protection is best achieved by developing active immunity and natural resistance, this is not yet possible in many instances. If exposure to infection cannot be avoided, protection in some of these instances may be conferred artificially by giving antibiotic substances, such protection lasting for only so long as adequate blood or surface levels are maintained. There is little difference between those substances originally called antibiotic because of our ignorance of their structure and mechanism, such as penicillin, and others such as sulphonamides, more generally designated chemotherapeutic substances. Both classes of drug interfere more with the parasite's mode of life than with that of the host and it is convenient to consider them together.

In this review I shall first consider in general the circumstances which warrant such protection, the hazards entailed, and then the practical details, taking as chief example the field where such prophylaxis was first used, the prevention of streptococcal infection in rheumatic fever patients, and touching rather more briefly upon other fields.

1. Circumstances Warranting Prophylaxis

There are two situations where antibiotic prophylaxis is of value, provided of course that a specifically effective antibiotic is available:

i. in healthy persons under circumstances where there is increased risk of infection; and
ii. in disease of various sorts where there is increased risk of infection due to either localized or general susceptibility.

i. The first situation is seen typically during an epidemic, when the organism, e.g., streptococcus, is prevalent and spreading rapidly. Such waves of infection often occur in small segregated communities, such as schools, camps or hospitals under conditions of overcrowding or bad hygiene. Treatment of infected persons and carriers and prophylactic antibiotic therapy of the rest of the community will usually halt such an epidemic. Adequate control has been obtained in all instances where a specific antibiotic is available for a particular organism without rapidly developing drug resistance. Thus in a meningococcal epidemic Kuhns, Nelson, Feldman & Kuhn (1943) were able with sulphadiazine to reduce the infection-rate from 40 cases in 18,800 untreated controls to 2 cases in 15,000 treated persons. However, as Phair & Schoenbach (1944) remark, the ordinary prevalence level of the community is rapidly regained when the epidemic ends. Regular prophylaxis, either systemic (Guthe, 1958) or with vaginal tablets (Ohno, Kato, Nagata, Hattori & Kanekawa, 1958), has also been employed in several countries with benefit both in professional prostitutes and others exposed to syphilis and gonorrhoea. In American sailors, the incidence of gonorrhoea in a controlled trial was reduced by a single oral dose of 100,000 units of penicillin taken after shore leave from 11.9 per 1,000 leaves to 1.8 (Eagle, Gude, Beckmann, Mast, Sapero & Shindledecker, 1948). Some types of bacillary dysentery may also be controlled by this means, using insoluble or soluble sulfa drugs. However, in the experience of Cheever (1946) the attack-rate in men treated with 4 g. sulphadiazine per day for six weeks was not significantly less (20%) than in controls (28%), perhaps because of drug-resistant organisms.

ii. The second situation needing prophylaxis is where a particular person must be protected often against commonplace organisms because of localized or general disease or constitutional abnormality. These conditions may be broadly grouped under:

a. infections (measles, pertussis, varicella, influenza and the common cold) where secondary infection is often potentially more harmful than the primary disease;
b. comatose or paralytic states, where aspiration bronchopneumonia is to be feared, as in cerebral vascular accidents, "respiratory" poliomyelitis, tetanus, congestive heart failure;
c. chronic disease prone to secondary lung or skin infection such as mucoviscidosis, diabetes, asthma, chronic bronchitis, bronchiectasis;
d. surgical conditions such as burns, parturition, catheterization, and others (dealt with separately).

Prophylaxis with antibiotics in (a), (b) and (c) is much less certainly of value than in the first situation: primarily because no antibiotic has a sufficiently broad spectrum to protect against all potential invaders and may quickly produce a floral change to more resistant types.

Thus, while it is generally agreed that otitis media is now much less common after measles than it used to be, it is not yet clear that this is owing to the prophylactic use of antibiotics. Karelitz, Chang & Matthews (1954) reduced the incidence of bacterial complications in measles from 27.6% in 81 untreated cases to 0–3% in 3 similar series treated according to various schedules of intramuscular penicillin. Sulfathiazole will not do this and indeed appeared in a controlled study to increase the incidence of otitis media and bronchopneumonia (Gibel & Litvak, 1942). Moreover, antibiotics as ordinarily given by the family doctor before admission to hospital led to an incidence in 130 cases of 30% with secondary bacterial invasion compared with an incidence of 15% in 298 patients

who had not been so "protected" (Weinstein, 1955b). As the latter author comments in a similar study of prophylactic antibiotics in pertussis and varicella (Weinstein, Seltser & Marrow, 1951), good nursing and the early recognition and prompt treatment of septic complications is more important than antibiotics which may alter the type of complication but not often their incidence. Oxytetracycline, however, has been found by Ritchie (1959) to be of value in preventing secondary infection after the common cold, since 1 g. per day for 2–3 days led to a reduction from 26% absences from work in untreated controls to 10% in the treated workers.

In comatose and paralytic states, antibiotics are widely used, but Petersdorf, Curtin, Hoeprich, Peeler & Bennett (1957), in a controlled study of 62 patients in coma but without evident infection on admission, found that pneumonia, skin infections and deaths were all more numerous in the treated group and concluded that antibiotic prophylaxis was of no benefit and distinctly hazardous in comatose patients. Similar conclusions were reached by Weinstein (1955a) in regard to "respiratory" poliomyelitis where secondary infections were three times more common in a group given chemoprophylaxis. Similarly, Lepper, Simon & Marienfeld (1957) found prophylactic antibiotics used singly or together of no real value in poliomyelitis cases with tracheotomy; they are, however, often given, as also in tetanus (Woolmer, 1955; Batten, 1956; Kelly & Laurence, 1956). In diabetes and congestive heart failure, McVay, Sprunt & Stern (1953) and McVay, Sprunt, Stern, Tatum & Lipscomb (1954) have found chlortetracycline (0.5 g. per day) of benefit in preventing infection in double-blind trials[1] lasting 20 months. McVay & Sprunt (1953), in a similar double-blind trial of chlortetracycline in chronic bronchitis, found decreased infection and duration of the stay in hospital, with increase in weight and haemoglobin and decrease in erythrocyte sedimentation rate, accompanied by only minimal side-effects.

Although sulphonamides are of no value in chronic bronchial infections (Kilpatrick & Oldham, 1954), oxytetracycline or tetracycline (Buchanan, Buchanan, Melrose, McGuinness & Price, 1958) appears to be of considerable value in carefully conducted trials over long periods (Helm, May & Livingstone, 1954; May & Oswald, 1956; Edwards, Buckley, Fear, Williamson & Zinnemann, 1957; Edwards & Fear, 1958), with little trouble on account of toxicity, drug resistance or superinfection. However, of 24 patients treated with oxytetracycline only 8 were still taking it between one-and-a-half and two-and-three-quarter years later (Helm, May & Livingstone, 1956). Similarly, oxytetracycline seems to be the best drug to use in severe bronchiectasis (Medical Research Council, 1957), and chlortetracycline has been found useful in mucoviscidosis (Shwachman, 1951). Oral penicillin given in doses of 120 mg. per day does not appear to be effective in asthmatic children (Lewis-Faning & Davies, 1959).

Another good example of a localized abnormality needing prophylactic care is the scarred valve of rheumatic heart disease or the susceptible heart with a congenital lesion: it is mandatory to protect these patients during such invasions of the blood stream by viridans organisms as have been shown to occur during the removal of teeth or infected tonsils and which may lead to subacute bacterial endocarditis. While antibiotic given before tooth extraction will to some extent reduce the number of cases showing positive blood cultures, especially in those with infected gums—41% compared with 79% of the unprotected (Glaser, Dankner, Mathes & Harford, 1948)—this benefit is not seen with multiple extractions, and the main value in cases with heart disease is probably the prevention of implantation. Since the peak of bacteriaemia is immediately after extraction, maximal levels are needed then and penicillin should be given not more than one hour preoperatively. If an antibiotic is given for a longer period before operation, a possible though apparently rare danger is the implantation of drug-resistant types (Cates, Christie & Garrod, 1951).

It is, however, with constitutional susceptibility that prophylaxis has been found to be of greatest value, and it is in this field that its use has been most thoroughly explored. Patients who have once had rheumatic fever are more than usually liable to a recurrence when they develop (as we all do from time to time) a β-haemolytic group A streptococcal throat infection. These infections can be prevented by regular daily prophylaxis (Thomas & France, 1939). Without such infection, there is no recurrence and no further cardiac damage, although the evolution of rheumatic heart disease from the damage of the first attack will proceed ineluctably in those with marked involvement.

2. Hazards and Drawbacks of Prophylactic Antibiotics

Drug toxicity is fortunately not usually a problem with the relatively small dosage necessary for prophylactic purposes, but sensitization, although uncommon, does unfortunately occur with most antibiotics. As in curative treatment, this is a hazard that has to be met. If a patient gives a history of sensitivity, either to drugs or manifested as asthma, urticaria, hay-fever, and so forth, it is wise to proceed very cautiously, and doubly so if he has previously received the proposed drug in curative dosage or by skin exposure. While the proportion of the population who have at some time received antibiotic treatment is not as high in Britain as in the United States of America[2] (despite a National Health Service!), reactivity is increasingly common. This may be due to previous local or systemic treatment, to handling penicillin or to the ingestion of milk from cows treated by penicillin for mastitis (Zimmerman, 1959). Sensitivity has also been seen, owing to the penicillin incorporated in poliomyelitis vaccine (Pelner, 1959). Thus, with an allergic reaction rate which may be as high as 18.6% following prophylactic intramuscular benzathine penicillin G (Hsu & Evans, 1958), or, with one injection only, as low as 1.3% (McFarland, 1958), sensitivity is obviously a major drawback to parenteral prophylaxis. Most of these reactions consist of urticaria only and very few show other manifestations of serum sickness: they are less likely with oral prophylaxis than with parenteral administration (Levin, 1957) and respond to penicillinase (Spiegel, 1959). The anaphylactoid reaction is rarer still but responsible for more fatalities. It usually follows injection (Lewis, 1957), but has been recorded after oral administration (Maganzini, 1957), almost entirely in patients with previous exposure (Dales, 1957). It is wise to have noradrenaline and hydrocortisone injections available when first administering penicillin and to give the first dose orally. We prefer to start prophylaxis in rheumatic fever patients while the patient is still under observation in hospital, since the serum sickness type of

[1] Trials in which neither the patient nor the observer knows until treatment is complete which of two or more preparations is being administered.—Ed.

[2] Only 14% of a large series of young American female nurses had never been given antibiotics (Kinney, 1957).

reaction may not appear until between 5 and 14 days after the first administration (Guthe, Idsöe & Willcox, 1958).

Other drawbacks to antibiotic prophylaxis are (i) the cost and—if the intramuscular route is chosen—(ii) occasional local pain or even sciatic neuropathy may be seen (Scheinberg & Allensworth, 1957). Superinfections due to alteration of bacterial flora do not occur with prophylactic dosage of penicillin or sulfadiazine (Miller & Massell, 1956), and the development of penicillin-resistant strains has not been observed with streptococci, pneumococci, gonococci or treponemata. Sulphonamide-resistance is a possibility (Stokes, 1947), but we have not found the development of resistant or "superinfecting" organisms a problem in rheumatic fever prophylaxis, even in an institution. In domiciliary practice this is even less likely. (With broad-spectrum antibiotics, however, used for other diseases, superinfections are troublesome—see discussion above.)

Thus the main drawback in rheumatic fever prophylaxis is sensitivity. With prophylaxis started under close medical supervision, this has not proved troublesome in an experience of nine years with over 1,000 children with rheumatic fever: a few have shown sensitivity to sulphonamides (manifest as agranulocytosis and leucopenia—3 in 500 cases (Bywaters, Hallidie-Smith & Thomas, 1957)), and for them penicillin prophylaxis has been adopted. In a few who have shown an urticarial reaction to penicillin, the treatment has been changed to sulphonamide.

3. Practical Use of Antibiotics in Prevention of Rheumatic Fever and Rheumatic Heart Disease

There are four ways in which antibiotics are currently being used in the hope of preventing rheumatic fever and rheumatic heart disease:

i. For short periods during group A β-haemolytic streptococcal epidemics in closed communities. This will prevent first attacks of rheumatic fever and recurrent attacks, as well as reducing invalidity due to tonsillitis and cutting short the epidemic.

ii. For prolonged periods in patients who are known to show special susceptibility to rheumatic fever, either because they have already had an attack of rheumatic fever or chorea or because they come from a family tainted with rheumatic fever.

iii. In large bactericidal doses for a period of ten days in patients who are known to be susceptible to rheumatic fever and who have developed a throat infection with group A β-haemolytic streptococcus. This treatment will usually eradicate the organism and, if started early enough, will prevent the later appearance of rheumatic fever and of the usual antibody response.

iv. A similar bactericidal course but lasting six weeks is being used in established rheumatic fever in the hope that the later development of rheumatic heart disease may be modified or abolished. Although, in the early days, penicillin was found not to affect established rheumatic fever, Mortimer, Rammelkamp and their colleagues (Mortimer, Vaisman, Vignau, Guasch, Schuster, Rakita, Krause, Roberts & Rammelkamp, 1959) have adduced some preliminary data which suggest that a course of penicillin which completely eradicates the organism, followed by the usual prophylaxis, may possibly diminish the cardiac sequelae. This, if confirmed, would be a most important advance, since current methods of treatment applied after rheumatic fever has developed do not appear to produce any spectacular diminution of later rheumatic heart disease.

In the control of epidemic streptococcal sore throat, penicillin is the drug of choice and may be given orally as phenoxymethylpenicillin (penicillin V) (120 mg. twice daily), or penicillin G (200,000 units twice daily), to those who are not infected: this must be combined with bactericidal treatment of infected persons and carriers. Alternatively, one single intramuscular injection of benzathine penicillin G (1.2 million units) will confer protection for a period of about one month: it is preferable, if there is any doubt that regular oral prophylaxis will be maintained (Schreier, Hockett & Seal, 1958).

Rheumatic fever recurrences are best prevented by oral crystalline penicillin G (200,000 units twice daily) or phenoxymethylpenicillin (120 mg. twice daily), as recommended by the Rheumatic Fever Committee of the Royal College of Physicians of London, July 1957 (Royal College of Physicians of London, 1957) and by the World Health Organization Expert Committee, 1957 (World Health Organization, 1957). Alternatively, if oral prophylaxis is not possible or its maintenance dubious, 1.5 million units of benzathine penicillin each month can be given. We have ourselves used sulfadiazine or triple sulphonamide (0.5 g.) for many years successfully: the recurrence rate in a group of 86 protected children studied over a five-year period was 1.2% per patient-year, compared with 5.6% in the unprotected group of 92 children (Bywaters & Thomas, 1958). Sulphafurazole has been equally satisfactory in other hands. The newer sulphamethoxypyridazine maintains protective levels for a longer period with a dosage of 1–2 g. twice a week only (Lepper *et al.* 1957), but there seems to be little other advantage, and serious sensitization has already been reported (Blanchard & Mertens, 1958).

This method of preventing recurrences of rheumatic fever is now 20 years old and has proved its value conclusively. It is not yet adequately employed in many parts of the world. Even when such a regime is prescribed it is not always adhered to: streptococcal infections occur, leading to recurrences and further heart damage. The reasons for this are sometimes parental (laziness, ignorance and lack of supervision) and sometimes due to failure on the part of the physician to realize the importance of this measure (Bywaters *et al.* 1957). Preventive measures always need a lot of reinforcement since there is not the immediate stimulus involved in curative measures: without constant reinforcement, any learnt act will, with the passage of time, tend to be omitted. If a susceptible patient is seen with sore throat or tonsillitis, he should immediately be treated with an intensive ten-day course of bactericidal penicillin, as advocated first by Massell, Dow & Jones (1948) and later by Denny, Wannamaker, Brink, Rammelkamp & Custer (1950). This will not always prevent recurrences but, if given early, will eliminate a large proportion of them. Sulphonamides are of no value, and inadequate dosage of antibiotic which fails to eliminate the organism is also a cause of therapeutic failure (Catanzaro, Rammelkamp & Chamovitz, 1958). Because many streptococcal infections are undetected by the parents and never seen by the family physician, this method of prevention of recurrences is not to be depended on as the sole measure. It should be used when a susceptible patient has developed a streptococcal infection, in spite of regular daily prophylaxis or for lack of it.

Prophylaxis should be maintained for as long as special risk and susceptibility exists: we have not found a diminution of recurrence rate in the first five years after an attack of rheumatic fever, but the risk undoubtedly decreases after this period. Increased risk of streptococcal infection occurs in schools, camps and barracks. Protection should be given in most instances until adult life is reached, and resumed if undue exposure is envisaged.

In these various ways, antibiotics, first introduced for the treatment of disease and developed rapidly in the Second World War for this purpose, can contribute even more to the prevention of disease, which is the ultimate aim of medical science.

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PREVENTIVE USE OF ANTIBIOTICS IN SURGERY

GERARD W. TAYLOR M.S. F.R.C.S.

Surgical Professorial Unit
St Bartholomew's Hospital, London

1 Circumstances in which bacterial contamination of a wound is unavoidable
  a Traumatic lacerations
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  c Operations on the urinary tract
  d Pulmonary surgery
2 Burns
3 Thigh amputation in ischaemic disease of the leg
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Preventive use of antibiotics is by nature speculative and there is greater opportunity for their misuse in this application than in therapy for established disease. In general, preventive administration should be restricted to situations carrying a real risk of infection. In this review it is not proposed to list every circumstance calling for the prophylactic administration of antibiotics but to underline the principles governing this use of these agents.

1. Circumstances in which Bacterial Contamination of a Wound is Unavoidable

a. Traumatic Lacerations

Wounds caused by trauma are contaminated by bacteria from the moment of their inception. Early and adequate surgical treatment is the most important factor in the prevention of wound sepsis, but experience with war injuries has demonstrated the additional benefit conferred by prophylactic antibiotic administration. This was clearly illustrated by the decreased incidence of gas gangrene which followed the introduction of penicillin in the later stages of the Second World War. In the Korean campaign evacuation of casualties was generally very rapid and the combination of early surgical treatment and antibiotic administration reduced the incidence of gas gangrene to a very low figure. Lindberg, Wetzler, Marshall & Newton (1955) investigated the bacterial flora of battle wounds in the Korean campaign and found that pathogenic clostridia were present in the wounds of 44% of patients at the time of initial débridement. The incidence of gas gangrene was, however, only 0.08%, and similar figures have been reported by Howard & Inui (1954, 1955). The control of wound infection may also be important in the prevention of "irreversible shock". Fine, Schweinburg & Frank (1954) demonstrated loss of normal capacity to inhibit bacterial invasion in dogs shocked by bleeding, and Jacobs, Weizel, Gordon, Korman, Schweinburg, Frank & Fine (1954) showed that the administration of antibiotics significantly increased survival rate in dogs subjected to severe haemorrhage. In a recent experimental study Mansberger, Ochsner, Jacob, Oppenheimer & Gillette (1958) also showed that antibiotics (streptomycin and oral neomycin apart) were effective in prolonging survival time in goats subjected to gross trauma. In these latter experiments the hind-quarters of the animals were grossly injured by explosive and it is likely that the beneficial effect of the antibiotics was due to suppression of bacteria of the clostridia group. It would seem clear then that in gross trauma antibiotics administered prophylactically are of value in preventing infection with anaerobic organisms. There is less justification for antibiotic "cover" in minor superficial wounds of an incised type that are common in civilian practice. Adequate surgical treatment of such wounds is usually available with very little delay, and the systemic administration of antibiotics is probably unnecessary. There may, however, be a place for the use of a bactericidal antibiotic solution as a topical application in the management of relatively minor wounds in civilian practice. Bacitracin is effective if used in this way, but this method of prophylaxis needs further study and evaluation. Penicillin remains the antibiotic of choice in the treatment of severe traumatic wounds. Garrod (1958) investigated the activity of 16 antibiotics against 29 strains of 10 species of clostridia. The tetracycline group and penicillin showed the maximum inhibitory effect, and penicillin was considered to be both effective and convenient. Garrod recommended an initial dose of 400,000 units each of potassium and procaine penicillin and a similar injection at six-hourly intervals.

b. Operations on the Alimentary Tract

Major oral operations often involve wide opening of tissue planes or may be combined with extensive dissection in the neck. Contamination with mouth organisms is unavoidable in these circumstances. The bacterial flora is predominantly penicillin-sensitive, and penicillin administration is an adequate and wise precaution. Tonsillectomy or dental extraction in patients with cardiac lesions should also be covered by injections of soluble penicillin, starting shortly before the surgical intervention. Resection of the oesophagus involves risk of contamination by the same bacterial flora, and penicillin will provide satisfactory protection over the operative period. In general, antibiotics should not be needed in gastric surgery. The bacterial flora of the stomach is comparatively scanty although this may vary with the local disease process. Högman & Sahlin (1957) investigated the bacterial flora of the stomach in 155 patients with gastric lesions. Bacteria were absent in 74% of patients with duodenal ulcer, in 17% of patients with gastric ulcer and in only 7% of patients with carcinoma. It can be argued that, when hypochlorhydria accompanies gastric lesions, the more profuse bacterial flora should warrant the prescription of antibiotics at the time of operation. There is no evidence, in fact, to show that this is beneficial and published data on this question present the reverse view. Hasslinger (1956), reviewing a large series of gastrectomies, found a higher incidence of local and chest infections in patients given sulphonamides or antibiotics than in those given neither. McKittrick & Wheelock (1954), comparing results of a small series of gastric and other operations with and without antibiotic cover, also showed that patients not treated with antibiotics did better. Linder (1957) analysed the post-operative course in a large series of gastric operations and found that infection in the wound and in the lung occurred slightly more frequently in those patients who had been given

antibiotics. These studies demonstrate that antibiotics used in a prophylactic sense not only do not confer benefit in this type of operation, but may even influence the post-operative course unfavourably.

Small intestine. The same considerations apply to operation on the small intestine, and the majority of patients requiring resection of small bowel should not need the administration of antibiotics. It has been shown, however (Cohn, 1956), that the administration of antimicrobial agents greatly facilitates recovery in those whose small bowel has been subjected to strangulation. The wall of damaged semiviable bowel is readily invaded by bacteria which complete the destructive effect of ischaemia. If the bacterial invasion can be checked by antibiotic administration, the collateral circulation is given time to become established. This may be important clinically if a decision is made to conserve damaged but potentially viable intestine and particularly if mesenteric thrombosis is being treated conservatively. The local use of neomycin (see below) may be the most appropriate method of antibiotic administration under these circumstances.

Operations on the colon. The bacterial flora of the large intestine is profuse and potentially more dangerous than that of the upper alimentary tract, and the post-operative mortality due to sepsis is higher after colonic operations than after those on any other surgical site (Foster, Deitch & Dadey, 1957). Most, but not all, surgeons would agree that it is wise to reduce the bacterial flora of the colon before operation. This view is supported by reports on the lowered incidence of infection and mortality rate after the introduction of sulphonamides as preparation for bowel operations (Dixon & Benson, 1944; Pemberton, Black & Maino, 1947). Simple mechanical cleansing will reduce the bacterial population of the colon considerably and this should always be done when purgation and colonic wash-outs are permissible. A further reduction of bacterial content can be obtained by the use of antimicrobial agents. Suitable sulphonamide preparations are effective and so are most of the broad-spectrum antibiotics. The disadvantage of antibiotic mixtures such as penicillin and streptomycin or the tetracycline group is that resistant organisms may rapidly repopulate the intestine, and a dangerous superinfection may arise. Staphylococcal enterocolitis has been reported frequently in association with the use of these agents in colonic surgery. Of the antibiotics, neomycin is the most widely used for bowel preparation at the present time. This use of neomycin was investigated by Poth (1953) who demonstrated its wide range of activity and rapid bactericidal effect. Staphylococcal resistance to the drug does not occur. Less than 5% of ingested neomycin is absorbed from the bowel and in the absence of obstruction a 24-hour preparation with six 1-g. doses is sufficient to reduce the bacterial content of the bowel to negligible proportions. Neomycin makes the stool consistency more fluid and this helps toward the pre-operative mechanical cleansing. According to Poth, if an empty bowel can be obtained, the pre-operative use of neomycin will ensure a persistent reduction in bacterial flora for some 6–7 days following the operation. Unfortunately, an empty bowel denuded of its normal bacterial flora is liable to be invaded by other pathogens such as the staphylococcus. This will not occur while neomycin is being given but may happen after the administration of neomycin has been stopped and before the normal bacterial population returns. In this way staphylococcal enterocolitis sometimes follows the use of neomycin. The sulphonamides do not reduce the bacterial flora to such a degree, and the risk of superinfection is correspondingly less. In this respect they are safer in the preparation of patients for colonic surgery, provided that there is sufficient time for a comparatively lengthy pre-operative course. If operation cannot be delayed, then neomycin is the agent of choice. It should not generally be necessary to administer antibiotics prophylactically in the post-operative period if adequate pre-operative preparation has been carried out and if the surgical procedure is technically satisfactory. McKittrick & Wheelock (1954) showed that no benefit was conferred by the use of antibiotics in the post-operative period in the majority of patients undergoing colonic surgery. They did, however, give antibiotics to patients undergoing anterior resection of the rectum, or when the anastomosis was technically difficult. It may be desirable occasionally to reduce the bacterial flora of the intestine in patients undergoing emergency procedures. Poth (1953) suggested that intestinal lavage, at operations, with a 1% solution of neomycin should be used for this purpose. If this method is employed, spillage of excess neomycin solution into the peritoneal cavity must be avoided. Symptoms of toxicity including respiratory failure may follow if more than 100 ml. of a 1% solution are allowed to escape into the peritoneal cavity (Doremus, 1959). Reduction in the bacterial population of the colon may also be beneficial in the surgical management of bleeding oesophageal varices in portal hypertension.

c. Operations on the Urinary Tract

Persistent infection in the urinary tract is almost invariably associated with local abnormality such as the presence of calculi or some defect of drainage. Surgical correction of these factors is required if the infection is to be eradicated permanently. Successful operative management may be facilitated by concomitant control of infection with antimicrobial agents. Prince (1946), using a sulphonamide and penicillin prophylactically, achieved a sterile urine post-operatively in about half of a series of patients undergoing prostatectomy. If infection is already present, the administration of antibiotics during the operative period may be regarded as prophylactic as well as therapeutic in that the risk of complication from sepsis may be reduced. The choice of antibiotic or chemotherapeutic agent must depend on careful sensitivity studies on the infecting organism.

d. Pulmonary Surgery

Operations involving division of lung tissue or bronchi may release pathogenic bacteria into the wound. This risk is greater if the primary pathological process for which the operation is being done is associated with infection, e.g., bronchiectasis. In this type of lesion antibiotic administration is indicated and the appropriate agent can be chosen by a pre-operative investigation of the sputum.

2. Burns

Injury by burning is virtually self-sterilizing at the moment of its inception, but after 24 hours contamination of the burned surface by a wide variety of bacteria is usual. Antibiotic therapy as a preventive measure against infection in burned patients has been studied carefully by the Burns Unit at the Birmingham Accident Hospital, and current practice in this respect is largely based on their findings. *Streptococcus pyogenes* is still the most frequent invader of the burned surface

and is more likely to cause delay in healing or failure of skin grafting than other bacteria (Lowbury, 1958). Fortunately, this streptococcus remains sensitive to penicillin, and Jackson, Lowbury & Topley (1951b) found that locally applied penicillin cream (1,000 units/g.) reduced the incidence of streptococcal infection from 38% to 1.7%. Sensitivity reactions to penicillin used in this way in burned patients are fortunately rare and occurred in only 6 of 1,232 patients observed by Davies (1958). Penicillin administered systemically, on the other hand, is not so successful in preventing colonization of the burned surface with haemolytic streptococci. This is because the lower local concentration of the systemically administered drug is reduced to ineffective levels by penicillinase-producing bacteria in the burned surface (Cruickshank & Lowbury, 1953). Burns in certain anatomical areas are often best managed by the exposure method in which dressings are not used and the burned surface is protected by an eschar formed by the natural exudate. Penicillin powder applied to the burn is effective in preventing streptococcal infection and also helps in the formation of a dry protective covering. Burns are notoriously susceptible to cross-infection and Colebrook, Duncan & Ross (1948) tried to prevent this by a regime which included chemoprophylaxis with penicillin cream and careful dressing techniques. This was successful in reducing the incidence of streptococcal infection, but colonization with staphylococci, *Proteus* and *Pseudomonas pyocyanea* was still a problem. Chemoprophylaxis against these latter invaders has proved disappointing. Lowbury, Topley & Hood (1952) tried to prevent staphylococcal infection by the systemic administration of antibiotics. This was initially successful but the rapid emergence of resistant strains led to the abandonment of the method. Staphylococcal resistance to neomycin and bacitracin does not occur, however, and Lowbury (1955) in a later trial found that significant protection against this organism was obtained with the local use of a cream containing neomycin (2 mg./g.) and chlorhexidine dihydrochloride (1 mg./g.). Infection with *Ps. pyocyanea* is particularly undesirable in a burn because of the lethal action of the blue pigment pyocyanin on the skin cells. Polymyxin is effective against these bacteria but is too toxic for routine systemic use. Topical application is successful, however, and Jackson, Lowbury & Topley (1951a), by adding polymyxin E (1 mg./g.) to the basic penicillin cream, were able to reduce the incidence of *Ps. pyocyanea* infection from 24% to 7%. This trial supports the use of locally applied polymyxin as a prophylactic against infection with *Ps. pyocyanea*, but at the present time the cost of this antibiotic is prohibitive. Jackson (personal communication) estimates that the cost of one such dressing for an extensive burn would be £25, and for this reason reserves its use for therapeutic purposes (as opposed to prophylactic use) in a heavily contaminated burn in which skin grafting is contemplated.

Current practice can be summarized thus. Chemoprophylaxis in the burned patient is desirable and successful against streptococcal invasion and is best effected by local application of penicillin cream (1,000 units/g. in lanette wax). Systemically administered antibiotics fail to control staphylococcal colonization because of the ready emergence of resistant strains. Rigorous control of cross-infection must remain the chief protection against infection with staphylococci and other common invaders. Infection by staphylococci and *Ps. pyocyanea* can be controlled with locally applied neomycin or polymyxin, but these agents are too costly for routine use in this way. They may, however, be indicated in large burns in which early and successful skin grafting is mandatory.

3. Thigh Amputation in Ischaemic Disease of the Leg

Mid-thigh amputation in patients with obliterative disease of their peripheral arteries may be complicated by gas gangrene. In a bedfast patient the perianal bacterial flora extends distally to include the thighs, and anaerobic bacteria can be recovered constantly from the skin of this area. Garrod (unpublished data) has shown that antiseptics, including iodine, applied to the skin are ineffective against these spore-bearing organisms. Contamination of the amputation wound with clostridia is therefore likely, and the ischaemic nature of the primary disease may favour the development of gas gangrene. Prophylaxis against this complication should be carried out with soluble penicillin (500,000 units) administered six-hourly.

4. Insertion of Foreign Material in the Tissues

The presence of foreign material in a wound substantially decreases local resistance to infection. It is also well known that, once sepsis is established around a foreign body, it will usually persist until the foreign material is extruded or removed. This propensity of foreign substances to potentiate infection seems to be related more to the gross structure of the materials than to their chemical properties. Thus, impervious monofilament sutures are less prone to septic complications than are porous materials of woven construction. Among the most frequently used foreign materials in modern surgical practice are homogenous tissues, taken at post-mortem and stored in a variety of ways. Bone grafts and blood vessels are often used in this way and sepsis sometimes complicates their use. Plastic arterial prostheses produce little or no reaction in the tissues but may also potentiate infection. This serious complication of arterial surgery has been the subject of a recent report which described instances where uncontrollable sepsis led to the removal of the arterial prosthesis and eventual sacrifice of the limb (Schramel & Creech, 1959). Rigorous asepsis must remain the chief defence against this type of disaster, but the consequences of infection may be so serious that the use of prophylactic antibiotics is justified. The choice of antibiotic is not easy, but a combination of penicillin and streptomycin is an effective bactericidal mixture and is less likely to produce undesirable sequelae than the chlortetracycline group.

5. Operations on the Heart and Brain

Antibiotics are frequently used in a prophylactic sense in operations on the heart and in prolonged neurosurgical procedures. Several authorities have agreed that antibiotic administration is wise in these circumstances (Foster *et al.* 1957; Linder, 1957; Altemeier, Culbertson & Vetto, 1955; Meleney & Johnson, 1953), although there is no published evidence to suggest that the incidence of infection is reduced by such means. In lengthy neurosurgical procedures, there is clearly greater opportunity for contamination of the wound by air-borne bacteria, and topical application of antibiotic solution has been recommended to counteract this. Teng & Meleney (1953) reported favourably on bacitracin used in this way and found that the solution had no harmful effect on exposed brain tissue.

6. Discussion

In general, antibiotics should not be used prophylactically in clean surgical procedures. It is misuse of these valuable agents if they are prescribed for fear of litigation or to counter poor surgical technique. There is no evidence to show that the incidence of infection is reduced by their use and in fact such studies as have been made tend to indicate that needless antibiotic administration may be harmful. Tachdjian & Compere (1957), in an evaluation of the use of prophylactic antibiotics, studied a group of 3,000 patients in whom clean orthopaedic operations had been done. Antibiotics were given prophylactically to 1,900 patients, while the remaining comparable 1,100 patients had no such treatment. There were twice as many infections, both major and minor, in the group receiving antibiotics and 3.6% of patients in this group suffered from complications directly related to the use of antimicrobial agents. It was also observed that major infections occurring in patients receiving antibiotics were more difficult to manage and that a considerable proportion of these infections were difficult to detect clinically until they had become well established. Conversely, major infection in the untreated patients declared itself promptly and responded well to drainage and treatment with the appropriate antibiotic. Barnes, Pace, Trump & Ellison (1959) have recently reported a similar study on general surgical patients, and also found that the sepsis rate was slightly higher in patients receiving antibiotics. Other workers have also reported on the failure of antibiotics administered prophylactically to reduce the incidence of post-operative sepsis and pulmonary infections (Högman & Sahlin, 1957; Linder, 1957; McKittrick & Wheelock, 1954).

Indiscriminate use of the majority of systemically administered antibiotics is followed by a sharp rise in the local population of resistant bacteria. The resistant staphylococcus is currently the chief cause of serious wound infection, and its predominance in surgical wounds is often related to the past use of antibiotics in that particular environment. Many reports have been concerned with the problem of staphylococcal sepsis, and most stress the need for the careful prescribing of antibiotics and the avoidance of prophylactic administration in clean surgical procedures (Wolma & Lynch, 1959; Brown, 1958; Burnett, Caswell, Schreck, Carrington, Learner, Steel, Tyson & Wright, 1958; Adams, 1957). Howe (1957) followed the staphylococcal infection-rate over an eight-year period and noted that it increased significantly over the years 1949–53 when antibiotics were being used heavily for prophylactic reasons. Antibiotics may also produce undesirable and sometimes dangerous sequelae at the time of administration. Tachdjian & Compere (1957) noted toxic effects in 3.6% of surgical patients receiving antibiotics prophylactically, and Weinstein, Goldfield & Chong (1954) reported the development of superinfection in 2.1% of 3,000 patients receiving antibiotics. It can be fairly stated then that in clean surgical procedures the administration of antibiotics not only fails to reduce the incidence of infection but may cause serious iatrogenic complications, and will in the long run facilitate the breeding out of resistant bacteria. Wound infection remains a serious problem in surgical wards, but surgical opinion generally is moving toward a solution to this problem by closer attention to asepsis and surgical technique at the time of operation and avoidance of cross-infection in the post-operative period. It is a curious paradox that the introduction of antibiotics should have awakened a renewed interest in the basic principles of surgical craft.

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THE CHEMOTHERAPY OF TUBERCULOSIS

With Special Reference to Patients whose Bacilli are Resistant to the Standard Drugs

JOHN CROFTON M.D. F.R.C.P. F.R.C.P.Ed.

Department of Tuberculosis and Diseases of the Respiratory System
University of Edinburgh

1 Chemotherapy for tuberculous patients whose organisms are fully sensitive to streptomycin, *p*-aminosalicylic acid (PAS) and isoniazid
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  a Choice of drug combinations in patients with bacilli resistant to streptomycin, PAS or isoniazid
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The standard drugs for the treatment of tuberculosis are streptomycin, *p*-aminosalicylic acid (PAS) and isoniazid. Before discussing the problems of patients whose organisms are resistant to one or more of these drugs, it is proper to outline the methods of treatment which should be used in patients with fully sensitive bacilli.[1] For it is the experience of our group that, if the standard drugs are used in the proper way, drug-resistant organisms should emerge very seldom if at all (Crofton, 1958a; Ross, Horne, Grant & Crofton, 1958). This confidence extends to all patients, however severe their disease. Douglas & Horne (1956) have shown that the sputum can be converted to negative and kept so, at least for a number of years, even in patients with persistent giant cavities. The sterility of such cavities, when these are resected after prolonged periods of proper chemotherapy, has been demonstrated by Stewart, Turnbull & Macgregor (1956). In some cases there was not even any residual evidence of tuberculosis.

Provided that one can avoid the emergence of drug resistance in patients' bacilli, the drugs should continue to be effective indefinitely and it should theoretically be possible to sterilize the lesions, or at least so to reduce the bacillary population that the patients' defences are capable of coping with the small residuum.

1. Chemotherapy for Tuberculous Patients whose Organisms are fully Sensitive to Streptomycin, *p*-Aminosalicylic Acid (PAS) and Isoniazid

a. Pulmonary Tuberculosis

The object of using combinations of the standard drugs is to prevent the emergence of drug resistance. The larger the patient's population of tubercle bacilli, the larger will be the number of drug-resistant mutants; consequently the more careful must the physician be in adhering to the combinations of drugs known to be most effective in preventing resistance. Patients with large cavities and severe disease obviously have much larger populations and are at a greater risk in this respect. Nevertheless in patients with mild lesions the risk, although reduced, is still there and any carelessness by the doctor in prescribing, or carelessness by the patient in taking, the drugs may have grim consequences at least in some cases.

The best combinations of different pairs of the standard drugs ("good chemotherapy") were well established by the careful series of controlled comparative trials carried out by the Medical Research Council in Britain between 1948 and 1955. These showed not only the superiority of drug combinations over therapy by a single drug, but also the superiority, when combined with daily isoniazid, of streptomycin given daily over streptomycin given twice weekly (see Table I). There was also a much greater incidence of drug resistance when the dose of sodium PAS, given combined with daily streptomycin, was lowered from 20 g. to 10 g. or 5 g. (Daniels & Hill, 1952).

Although we have had practically uniform success in converting the sputum to negative by using streptomycin (1 g. daily) together with isoniazid (100 mg. twice a day), provided that the patient's bacilli were initially sensitive, we have had to modify this practice because of finding patients who had been primarily infected with tubercle bacilli resistant to streptomycin or occasionally to isoniazid (Thomas, Borthwick, Horne & Crofton, 1954; Murdoch & Grant, 1955). The survey of the Medical Research Council in Britain (Fox, Wiener, Mitchison, Selkon & Sutherland, 1957) has shown that over 5% of patients with newly diagnosed tuberculosis, recruited on a sample basis throughout the British Isles, have been infected with tubercle bacilli resistant to at least one of the standard drugs. A current international survey (Crofton & Rist, 1960) has shown a slightly higher figure. In consequence, it is our present practice to treat all hospital patients with all three drugs until it is known that their bacilli were initially sensitive.

"Good chemotherapy" can be summarized as follows:

i. Hospital patients under age 40. Streptomycin (1 g. daily) plus sodium PAS (5 g. three times daily) plus isoniazid (100 mg. twice daily). When it is known that the pre-treatment cultures were fully sensitive we usually drop the PAS while the patient is in hospital, continuing with daily streptomycin and isoniazid.

ii. Hospital patients over age 40. In such patients there is a risk of vestibular disturbance with daily streptomycin (1 g.) and we have normally given streptomycin (1 g. three times weekly) plus sodium PAS (5 g. three times daily) plus isoniazid (100 mg. twice a day). Theoretically there might be a slight risk of drug resistance with this combination, for if the patient's organisms were primarily PAS-resistant he would be receiving intermittent streptomycin with daily isoniazid and there would be a slight risk of the emergence of isoniazid-resistance followed by streptomycin-resistance. Although we have treated several hundred patients with this combination, we have not yet seen resistance emerge. The risk must therefore be small. Possibly it might be reduced further by a daily dose of 0.5 g. streptomycin, but we have no direct evidence that this would be more reliable.

[1] A number of the *British Medical Bulletin* on tuberculosis contains an account by Scadding (*Brit. med. Bull.* 1954, **10**, 135) of the use of antibiotics and other drugs available for treating this disease at that time; and one by Fox (*Brit. med. Bull.* 1954, **10**, 108) on the conduct of controlled trials of such treatment. The subject was also discussed by Daniels (*Brit. med. Bull.* 1951, **7**, 320).—ED.

TABLE I. Medical Research Council's Controlled Trials: Drug Resistance with Various Drug Combinations

Drug combinations	Type of resistance	After 3 months				After 6 months			
		Number of patients	Positive cultures	Patients with resistance (%)	Positive cultures resistant (%)	Number of patients	Positive cultures	Patients with resistance (%)	Positive cultures resistant (%)
Sm alone (2 g. daily)[1]	Sm†	42	?	71	?	—	—	—	—
Sm alone (1 g. daily)[2]	Sm	53	47	66	74	43	34	63	79
Sm (1 g. daily) + NaPAS (5 g. 4 times daily)[2]	Sm	48	33	8	12	43	28	7	11
Sm (1 g. daily) + NaPAS (5 g. 4 times daily)[3]	Sm	—	55	—	7	—	58	—	6
Sm (1 g. daily) + NaPAS (1.25 g. or 2.5 g. 4 times daily)[3]	Sm	—	43	—	19	—	45	—	34
Sm (1 g. daily) + NaPAS (5 g. 4 times daily)[4]	Sm	219	68	2	6	44	7	5	(29)
Sm (1 g. daily) + INH (100 mg. twice daily)[4]	Sm	120	26	2	8	58	4	0	(0)
Sm (1 g. daily) + INH (100 mg. twice daily)[5]	Sm	74	18	0	(0)	—	—	—	—
Sm (1 g. daily) + INH (100 mg. twice daily)[6]	Sm	105	22	0	(0)	60	2	0	(0)
Sm (1 g. twice weekly) + INH (100 mg. twice daily)[5]	Sm	94	22	3	(14)	—	—	—	—
Sm (1 g. twice weekly) + INH (100 mg. twice daily)[6]	Sm	132	31	2	10	64	9	5	(33)
NaPAS alone (5 g. 4 times daily)[2]	PAS†	—	—	—	—	37	?	32*	?
NaPAS (5 g. 4 times daily) + Sm (1 g. daily)[2]	PAS	—	—	—	—	30	?	7	?
NaPAS (5 g. 4 times daily) + INH (100 mg. twice daily)[5]	PAS	67	18	1	(6)	—	—	—	—
NaPAS (5 g. 4 times daily) + INH (100 mg. twice daily)[6]	PAS	92	22	1	(5)	47	0	0	(0)
NaPAS (5 g. twice daily) + INH (100 mg. twice daily)[5]	PAS	65	14	2	(7)	—	—	—	—
NaPAS (5 g. twice daily) + INH (100 mg. twice daily)[6]	PAS	97	23	1	(4)	27	2	0	(0)
INH alone (100 mg. twice daily)[4]	INH†	243	132	35	64	48	30	58	93
INH (100 mg. twice daily) + Sm (1 g. daily)[4]	INH	120	28	2	11	58	4	3	(50)
INH (100 mg. twice daily) + Sm (1 g. daily)[6]	INH	105	22	2	(9)	60	2	0	(0)
INH (100 mg. twice daily) + Sm (1 g. twice weekly)[6]	INH	132	30	9	40	64	9	13	(89)
INH (100 mg. twice daily) + NaPAS (5 g. 4 times daily)[6]	INH	92	24	0	(0)	47	0	0	(0)
INH (100 mg. twice daily) + NaPAS (5 g. twice daily)[6]	INH	97	25	2	8	27	2	0	(0)

* Within 6 months: times and positive cultures not stated

† Sm: streptomycin; NaPAS: sodium PAS; INH: isoniazid

Percentages in parentheses refer to totals of less than 25.

Data derived from the following reports:

1 Medical Research Council (1948) *Brit. med. J.* **2,** 769
2 Medical Research Council (1950) *Brit. med. J.* **2,** 1073
3 Daniels, M. & Hill, A. B. (1952) *Brit. med. J.* **1,** 1162
4 Medical Research Council (1953) *Lancet,* **2,** 217
5 Medical Research Council (1953) *Brit. med. J.* **2,** 1005
6 Medical Research Council (1955) *Brit. med. J.* **1,** 435

iii. Mild cases treated as out-patients, and patients after discharge from hospital. It is convenient to treat such patients with oral drugs. Our standard practice is to give 5 g. sodium PAS together with 100 mg. isoniazid, both twice daily. Isoniazid and PAS are given in the same cachets or the same tablets so that the patient cannot take one without the other.

It has been shown elsewhere that some patients metabolize administered isoniazid very rapidly (Hughes, Schmidt & Biehl, 1955; Grosset, Grumbach & Canetti, 1957) and it has been suggested that in consequence the low dose of isoniazid given above would be inadequate for some patients. The uniform sputum conversion obtained in some British series (Ross *et al.* 1958; Crofton, 1958a) suggests that this objection is more theoretical than practical. It is not excluded that higher doses of isoniazid might result in more rapid improvement of the patients. A trial in the United States of America (Mount & Ferebee, 1954) suggested that a daily dose of 10 mg./kg. was no better than that of 3 mg./kg., although a recent French trial (Bernard, Jarniou, Israël, Enjalbert & Fabre, 1959) suggested that there might be some difference. A

current controlled trial by the Tuberculosis Society of Scotland is comparing 16 mg./kg. with the standard dose of 100 mg. twice daily. No results are yet available.

Duration of chemotherapy. Chemotherapy should be given for a sufficient length of time to prevent relapse in all cases. The results of Ross and his colleagues (Ross *et al.* 1958) in patients admitted in 1953 and followed for 3–4 years showed that there was a relatively high relapse rate in patients who received less than one year's chemotherapy but only one relapse among 136 patients treated for more than one year. There were no relapses in any patients who received more than 18 months of such treatment. A follow-up of the 1954 admissions to Edinburgh hospitals has once more shown no relapses in those with more than 18 months' chemotherapy (Ross, personal communication). These results received striking confirmation in the studies by Stewart and her colleagues of the isolation of tubercle bacilli from specimens of lung resected after varying periods of chemotherapy. There was a steadily decreasing positivity rate up to one year of chemotherapy. There were only two positive isolations from 101 lesions resected after more than one year of chemotherapy and none from 22 lesions from patients who had received more than 18 months of such therapy (Stewart *et al.* 1956, and private communication).

It seems therefore that, in all except the mildest cases, chemotherapy should be continued for 18 months and in more severe cases up to two years. At present there is no definite evidence that it is necessary to continue beyond two years, although studies comparing different periods of chemotherapy are being carried out at the present time by the Medical Research Council in Britain.

b. Other Forms of Tuberculosis

The principles of chemotherapy outlined above are applicable to all other forms of tuberculosis. There has been a tendency to give higher doses of isoniazid in tuberculous meningitis, and many clinicians give a daily dose of 10 mg./kg. It has not, however, been definitely established that this is necessary. In Great Britain the relative scarcity of tuberculous meningitis now makes controlled trials impracticable.

2. Reasons for Therapeutic Failure

Before going on to consider the problem of patients who have drug-resistant organisms, it is important to discuss briefly why we have to consider these at all. In our experience the main causes of failure are as follows:

i. Primary infection with drug-resistant organisms. If the patient's organisms are naturally resistant to one drug of a pair used to treat him, the bacilli are liable also to become resistant to the second member of the pair. We avoid this risk by giving all three drugs but we have had one or two unfortunate patients who have been primarily infected with organisms resistant to two of the three main drugs and whose bacilli have in consequence become resistant to the third before we had the results of the pre-treatment resistance tests.

ii. Failure to appreciate the likelihood of the presence of drug-resistant organisms in a patient who has had previous unsatisfactory chemotherapy. For instance, if the patient has ever had one drug alone or has ever had intermittent treatment with streptomycin combined with daily isoniazid or daily PAS, there is a risk that his organisms will be resistant to one or more of the drugs used. To use a third drug in combination with a possibly unreliable drug may result in resistance to the third drug also.

iii. Failure of out-patients to take the drugs as prescribed. The development of tests for PAS in the urine has shown that a high proportion of out-patients fail to take their prescribed drugs regularly (Simpson, 1956; Dixon, Stradling & Wootton, 1957; Fox, 1958). Even in hospital the nursing staff must ensure that every dose of the drug is not only received by the patient but actually seen to be swallowed.

iv. Insufficient duration of therapy.

All these causes of failure, except the last, are liable to result in the development of drug resistance. Even though PAS and isoniazid are given in the same cachet or tablet, a failure to take the full number in the day may result in the emergence of isoniazid-resistance (Simpson, 1959).

3. Treatment of Patients with Drug-Resistant Tubercle Bacilli

The high degree of success which follows the treatment of newly diagnosed patients with "good" chemotherapy makes it unethical to carry out trials of new drugs on such patients. New drugs have to be tried on patients whose organisms are resistant to the standard ones. If proper methods of chemotherapy have been generally used such patients should be very few. The scientific data on which to base one's treatment are therefore very much less satisfactory than the data obtained for "good" chemotherapy in the large-scale trials of the Medical Research Council in Britain referred to in section 1a.

In the following paragraphs brief notes will be given on some of the drugs available, followed by a review of possible drug combinations for particular types of case.

i. Viomycin. There is little doubt that viomycin has antituberculosis activity. Unfortunately it has toxic side-effects on the vestibular apparatus and on the kidneys. When used alone, drug resistance is common and may develop rapidly. It has been found that toxic reactions are rare when the drug is given in doses of 1 g. twice a day on two days in the week. In fact it is possible that we have been over-cautious and that we might get few toxic reactions if we used viomycin daily, perhaps in a dose of 1 g. Murdoch & Stewart (1956) have suggested that in most, but not all, cases viomycin-resistance can be prevented, or at least deferred for many months, by combining viomycin (1 g. twice daily on two days in the week) with tetracycline (2 g. twice daily). Subsequent experience has confirmed these early results. Lower doses of tetracycline result in a much higher incidence of viomycin-resistance. A number of successful results in otherwise obstinate cases have been recorded (Pines, 1957). Nevertheless, in our experience the antituberculosis effect is relatively weak and may often be insufficient to achieve sputum conversion in advanced cases. But the drug combination is almost always sufficient to prevent deterioration while the chemotherapy is continued, except in the few cases in which viomycin-resistance emerges.

ii. Tetracycline drugs. The tetracycline drugs have only very weak antituberculosis action, but there is more or less good evidence to suggest their value, when given in high dosage, in delaying or preventing the emergence of tubercle bacilli resistant to streptomycin (Miller, Walker, Dye & Tempel, 1952) and isoniazid (Stewart, Turnbull & Crofton, 1954). As already mentioned, we have found a great reduction in the incidence of viomycin-resistance (Murdoch & Stewart, 1956) and have had also satisfactory experience in the case of cycloserine (see below). With doses lower than 2 g. twice a

day there is a much higher incidence of resistance to the second drug. On the other hand, we have found tetracycline quite ineffective in preventing resistance to pyrazinamide (Stewart, Murdoch, Crofton & Hay, 1957) or to "1314" (Somner, Ross, MacLeod, Stewart, Horne & Crofton, 1959). Some diarrhoea is quite common in the first couple of weeks of taking tetracycline, but usually it then decreases and causes little trouble. Serious intolerance is unusual.

iii. Pyrazinamide. Pyrazinamide is a substance chemically resembling nicotinamide but with an additional nitrogen atom substituted in the ring. Earlier American work (for instance, Yeager, Munroe & Dessau, 1952) suggested that it was a powerful antituberculosis drug. Pyrazinamide has potential toxic effects on the liver, and deaths have occurred from acute liver atrophy. It should therefore be used only in hospital, at least initially, and liver function tests should be carried out at regular intervals. Should any abnormality be shown by the tests, it is wise to stop the drug, at least temporarily. The dosage we have used has been 40 mg./kg./day divided into two equal doses with a maximum of 1.5 g. twice a day. When given alone, drug resistance emerges rapidly in many cases, often within six weeks. Technically the resistance tests are difficult to carry out. Details are given by Stewart and her colleagues (Stewart *et al.* 1957). It has been claimed that pyrazinamide plus isoniazid is a highly effective combination in patients whose bacilli are initially fully sensitive to both drugs. Resistance seldom emerges to either of the drugs used. Our own limited experience confirms this. On the other hand, we have found no evidence to confirm the suggestion that pyrazinamide is more effective on bacilli of a low degree of isoniazid-resistance (Stewart *et al.* 1957; MacLeod, Hay & Stewart, 1959). Pyrazinamide-resistance has emerged in several of our patients treated with both drugs, but whose bacilli were initially slightly isoniazid-resistant. One failure even occurred in a patient whose bacilli had been previously isoniazid-resistant but had apparently reverted to full sensitivity. On the other hand, we have had no failure in the limited number of patients we have treated with both drugs and who have had neither previously.

It is uncertain whether streptomycin or PAS would be as effective as isoniazid in preventing the emergence of pyrazinamide-resistance. As already mentioned, we found tetracyclines ineffective. The value of cycloserine is still uncertain (see below).

iv. Cycloserine. Cycloserine has a definite antituberculosis action (see, for example, Livings, 1956; Ståhle, 1958; Weinberger, Lindars & Pasha, 1958). Most recent authors have suggested that the antituberculosis effect is relatively weak. Our own experience confirms this. In doses above 1 g. daily, toxic effects have been a serious problem and sometimes result even from that dose (Walker & Murdoch, 1957). The main toxic effects have been psychotic symptoms, usually starting within a few days of beginning treatment, and convulsions. Our group have had the impression that certain patients, on the verge of cor pulmonale, have been precipitated into cardiac failure (Walker & Murdoch, 1957). Because of the toxic effects some authors have recommended a maximum dose of 0.5 g. per day, but there is evidence that this smaller dose is even less effective (United States Public Health Service, 1956). It is thought that the toxic effects are less common if treatment is begun with a dose of 0.25 g. twice daily, gradually increased to a dose of 0.5 g. twice daily, raising the dosage every 4–7 days.

There is no clear evidence regarding the incidence of cycloserine-resistance, perhaps because the drug has often been given with a second agent. In our own experience cycloserine-resistant organisms have not emerged when the drug was combined with 2 g. tetracycline twice daily. Cycloserine has recently been used with pyrazinamide and with "1314", but so far there is no unequivocal evidence to show how effective it is in preventing resistance to these drugs. Certainly with pyrazinamide it seems that there will be at least some failures.

v. "1314" (α-ethylthio*iso*nicotinamide). This drug was evolved, and received its initial bacteriological and clinical trial, in France (Rist, Grumbach & Libermann, 1959; Brouet, Marche, Rist, Chevallier & LeMeur, 1959). It is a compound allied chemically to isoniazid, but there is no cross-resistance with isoniazid. That is to say, it is fully effective against isoniazid-resistant tubercle bacilli. Our group have been able to carry out a preliminary trial of the drug (Somner *et al.* 1959), attempting to prevent drug resistance by a combination with tetracycline (2 g. twice daily). By the technique of serial bacterial counts in the sputum, which we have used in the testing of a number of new drugs, we have shown that "1314" has a powerful antibacterial action. Unfortunately in most cases tetracycline failed to prevent the emergence of resistance. "1314" is given by the mouth and in most of the trials up to date has been used in a total daily dose of 1 g. Unfortunately in this dosage gastrointestinal intolerance has proved a serious problem. A double-blind trial[2] of intolerance to different doses (Somner, 1959) suggested that a daily total of 0.5 g., divided into two daily doses, will reduce but by no means abolish side-effects. It remains to be seen whether such a dose is effective in combination with pyrazinamide or with cycloserine. A trial of this kind is now being conducted by the Clinical Trials Committee of the British Tuberculosis Association.

a. Choice of Drug Combinations in Patients with Bacilli Resistant to Streptomycin, PAS or Isoniazid

It will be clear from the foregoing account that the newer antituberculosis drugs are in general less effective and more toxic than streptomycin, PAS and isoniazid given in proper combinations. They are usually also very much more expensive. Owing to the ethical problems of testing, we are very much less well informed about them. Suggestions regarding treatment, outlined in the following paragraphs, must be read with these limitations in mind.

i. The patient who has had previous "doubtful" chemotherapy. Many patients are seen who give a history of having had previous chemotherapy with combinations now known to be less uniformly effective in preventing drug resistance. The most common is intermittent streptomycin with daily isoniazid or PAS. It is rash to start such a patient on all three drugs. If his organisms are already resistant to two of them, they may well become resistant to the third also. Our general policy is to start such patients on viomycin (2 g.) in two divided doses on two days in the week together with tetracycline (2 g. twice daily). It is not highly effective therapy but it does prevent deterioration and allows the patient to hold his own, and indeed somewhat improve, while the clinician is awaiting the results of resistance tests. It is most important that such tests should be carried out on more than one specimen of sputum, especially if there is a possibility that the organisms

[2] A trial in which neither the patient nor the observer knows until treatment is complete which of two or more preparations is being administered.—Ed.

may be PAS-resistant. Three specimens should be a minimum. If any show drug resistance it is probable that the patient's organisms will behave clinically as if they were resistant.

The criteria for drug resistance given by different bacteriologists vary greatly. In our own work we have carefully correlated clinical outcome with the results of resistance tests (Crofton, 1957a, 1957b, 1958b). We have found that the lowest degree of detectable drug resistance may be clinically significant and that tests on a solid medium are essential in order to detect all such clinically significant resistance (Stewart, 1955). It is true that such tests may show drug resistance when a proportion of the patient's organisms are still sensitive. If the growth on the drug-containing tube is not equal to the growth on the control tube not containing the drug, it is possible that the drug in question may still be of some value in suppressing residual sensitive organisms. Nevertheless this drug should never in any circumstances be relied on to prevent resistance to a second drug. It will fail in a proportion of cases. It should therefore be given only in addition to another effective combination of drugs.

ii. The patient with tubercle bacilli resistant to streptomycin only. PAS plus isoniazid should be used. In severe cases it is wise to use the PAS in a dose of 5 g. of the sodium salt four times daily together with isoniazid (100 mg. twice a day). There is now some unpublished evidence to suggest that in severe cases there may be some failures and it is perhaps wise to add a third effective drug such as viomycin or pyrazinamide.

iii. The patient with tubercle bacilli resistant to PAS only. In a younger person the treatment would obviously be with 1 g. streptomycin daily combined with 100 mg. isoniazid twice daily. In older people this does give a risk of vestibular disturbance. If a case is a severe one it is probable that this risk should be taken. If not, the dose might be lowered to 0.5 g. or 0.75 g. daily. We have no unequivocal evidence that such a dose will not result in a few failures, but on the other hand it may be fully effective.

iv. The patient with organisms resistant to isoniazid only. The patient should be treated with daily streptomycin (1 g.) together with sodium PAS (5 g. four times daily). As already mentioned, it has been shown in the Medical Research Council trials that lower doses of PAS are much less effective in preventing resistance to streptomycin. The problem of streptomycin doses in older people is the same as in the case of the patient described in paragraph iii above.

v. The patient whose tubercle bacilli are resistant to both streptomycin and PAS. The combination of pyrazinamide (20 mg./kg.) with a maximum of 1.5 g. given twice daily, combined with isoniazid (100 mg. twice daily), is undoubtedly the most powerful. In severe cases the risk of pyrazinamide toxicity should probably be taken. In very mild cases it may be justifiable to use viomycin plus tetracycline.

vi. The patient whose tubercle bacilli are resistant to both PAS and isoniazid. In such cases the combination of streptomycin (1 g. daily) and pyrazinamide in the doses already given may well be the best, although there is very much less evidence about the value of streptomycin in preventing pyrazinamide-resistance. It might be wise to use in addition tetracycline (2 g. twice daily), or even cycloserine in a dose of 0.5 g. twice daily.

vii. The patient whose tubercle bacilli are resistant to both streptomycin and isoniazid. In such a patient we are left only with PAS among the standard drugs and this is the weakest of them. It is uncertain whether PAS would be adequate to prevent the development of pyrazinamide-resistance. The best treatment would be to combine PAS in the highest tolerated doses with pyrazinamide and also to use cycloserine or viomycin in the doses already given.

viii. The patient whose organisms are resistant to streptomycin, PAS and isoniazid. These patients present a formidable problem. Not only are their organisms resistant to the standard drugs but they usually have extensive chronic disease, inadequately treated over long periods. Viomycin plus tetracycline may make a few such patients fit for surgical operation but is unlikely to attain sputum conversion on its own. Cycloserine plus tetracycline is likely to have similar limitations as well as the relatively high risk of toxicity from cycloserine. It seems possible that cycloserine plus pyrazinamide may be a more effective treatment, but it would be wise to add viomycin (2 g. twice weekly) as well. We have as yet no adequate evidence as to the efficacy of such combinations although trials are being carried out at the present time. The value of "1314" combined with pyrazinamide or cycloserine is at present under trial.

b. The Place of Surgery in the Chemotherapeutic Programme

Although surgery is now relatively seldom required in patients whose organisms are sensitive to the standard drugs, chemotherapy is so much less reliable in patients whose organisms are resistant to the standard drugs that it should always be seriously considered. The aim should be to extirpate the major lesion containing the highest proportion of the patient's resistant population. This will not only remove the risk of the spread of these bacilli elsewhere in the lung but also, by reducing the population, reduce the risk of the emergence of drug resistance if the patient has to be treated with less effective chemotherapeutic combinations. The decision as to the optimal time for surgery is a very difficult one. There is no doubt that resection is much less likely to be complicated by the spread of infection if the patient's sputum has become culture-negative by the time of operation. On the other hand, there is always the risk that drug-resistant organisms may have emerged if the operation is put off too long, and then the infective complications of operation are likely to be even more serious. It is wise, therefore, if at all possible, to reserve one drug, preferably a powerful one such as pyrazinamide or "1314", to add to the previous combination as operation cover.

It is impossible to generalize as to the correct time for operation. If the chemotherapy used includes one of the more powerful drugs, it is likely that the patient's bacillary population will be substantially reduced after three months' chemotherapy. With most of the combinations outlined, resistant organisms are not very likely to have emerged by that time; but the trend of sputum positivity should be closely studied before a decision is made.

4. Conclusions

If proper combinations of the standard drugs are both prescribed by the doctor and taken by the patient, drug-resistant organisms should almost never emerge, and sputum conversion to negative should always be achieved except in the very rare cases unfortunate enough to have been primarily infected with tubercle bacilli resistant to two or more of the standard drugs. Nevertheless, owing sometimes to the

patient's errors or more often to the previous use of drug combinations now known to be less effective, some patients present with resistant organisms. Such a patient should be regarded as a challenge. Meticulous resistance tests should be carried out on a number of cultures and no potentially useful drug should ever be risked until it is certain both that his organisms are sensitive to it and that it is given in combination with other drugs which have the best chance of being effective in preventing the growth of resistant mutants. The whole programme for the individual case should be carefully planned.

If sufficient medical, bacteriological and surgical skill is available an important proportion of these unfortunate patients can be salvaged.

ACKNOWLEDGEMENTS

This article is based on the work of many colleagues in the Medical Research Council and in Edinburgh. Of the latter I am particularly grateful to Drs I. W. B. Grant, N. W. Horne, J. D. Ross, J. Williamson, Sheila Stewart and A. T. Wallace. Most of the research in Edinburgh was supported by grants from the Royal Victoria Hospital Tuberculosis Trust.

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THE TREATMENT OF BACTERIAL ENDOCARDITIS

A. ERIC DORMER M.D. M.R.C.P.

Medical Unit
St Bartholomew's Hospital, London

1 *Streptococcus viridans*
2 *Streptococcus faecalis*
3 *Staphylococcus*
4 Miscellaneous organisms
5 Value of newer antibiotics
  a Ristocetin
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7 Failure of treatment
8 Conclusion
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Despite the development of considerable resistance to the infecting organism as shown by the presence of circulating antibodies (Keefer, 1937), and by histological evidence of valve healing (Moore, 1946), spontaneous recovery from bacterial endocarditis is rare and not to be expected. However, well-documented cases of recovery are recorded (Blumer, 1923). Lichtman (1943), reviewing 2,596 cases, found a 1% survival in the pre-sulphonamide era, but significant improvement was not obtained until the introduction of penicillin. By far the commonest infecting organisms are viridans and allied streptococci. *Streptococcus faecalis* and staphylococci each cause some 5–15% of cases, while in a smaller number are found gram-negative organisms and a variety of miscellaneous organisms mentioned by Jones (1950). The broad principles underlying the management of bacterial endocarditis will be illustrated by tracing the development of antibiotic therapy for each group of organisms.

1. *Streptococcus viridans*

Basic research into the behaviour of penicillin demonstrated that it could penetrate the fibrin barrier (Nathanson & Liebhold, 1950), and there kill bacteria. Keefer (1953) observed that *Streptococcus viridans* behaved differently from pneumococci, being killed more slowly. Hamburger & Carleton (1955) have shown the importance of the size of bacterial population—penicillin taking longer to eliminate denser groups. Hunter & Paterson (1956) noted how easily blood clot could protect bacteria. All observations therefore point to the need for high dosage and prolonged treatment.

Initial successes with penicillin were reported by Loewe, Rosenblatt, Greene & Russell (1944) who cured seven patients, while Dawson & Hobby (1944) cured two, with total doses of 0.8 and 1.4 mega (million) units. Clinical application of penicillin before 1953, reported in many series, resulted in cure of from 55% to 72% of patients (see, for example, reports by Gorlin, Favour & Emery (1950) of 817 collected cases; Donzelot, Le Bozec, Kauffmann & Escalle (1953) of 202; Hueber & Saexinger (1950) of 100). One of the most important trials was organized by the Medical Research Council in Britain, the final report by Cates & Christie (1951) covering 442 cases. From this, recommendations were made that patients with penicillin-sensitive infections should receive a minimum of 2 mega units daily for 4–6 weeks, the penicillin being usually given at three-hourly intervals; for streptococci with more than four times the resistance of the standard Oxford staphylococcus the dose was increased to 4 mega units per day.

Modification of drug schedules has since then been largely left to individual effort. Use of larger doses of penicillin less frequently, e.g., 1 mega unit six-hourly, makes treatment less arduous and, since treatment with penicillin alone involves the least risk of toxic effects, can be advocated for sensitive infections. Attempts to shorten the duration by giving massive doses, e.g., 10 mega units per day for ten days (King, Schneierson, Sussman, Janowitz & Stollerman, 1949), were not sufficiently successful, although Hamburger & Stein (1949) cured eight of ten patients with 15 mega units per day for two weeks.

Eagle (1952) has indicated the importance of peak serum levels. Procaine penicillin results in much lower peak levels and more even concentration, but this preparation has been used successfully in several series, such as those of Roston & Stollerman (1949) using 0.6 mega units twice daily for some six weeks, and Hall, Dowling & Kellow (1955) who used 0.6 mega units four times a day in combination with streptomycin over two weeks.

Following Austrian reports in 1953 that oral penicillin V could lead to consistent and adequate blood levels, its use in bacterial endocarditis was reported. Quinn, Colville & Cox (1956) and Harris (1958) noted its successful use alone, and Quinn, Colville, Cox & Truant (1957) and Walker & Hamburger (1957) in combination. Most reports are of 1–2 mega units given four-hourly, but total daily doses range from 1.2 to 12.0 mega units. Average levels of 5.34–8.75 units/ml. serum, with range of 2.4–20.0 units/ml. at from one-half to four hours were obtained with doses of 2 mega units four-hourly. But with a similar dose Santos-Buch, Koenig & Rogers (1957), reporting on six cases, noted that serum levels varied widely in the same patient and between individuals, maxima of 1.2–51.2 and minima of 0.8–6.4 units/ml. being observed. In one patient levels were consistently below 1.2 units/ml. until probenecid was added. Highest levels are seen 2–4 hours following doses taken after food. While, therefore, for certain patients oral medication may be indicated and doses of 1–2 mega units 4–6-hourly may be sufficient, adequate blood levels cannot be guaranteed and may require checking. Nausea can interrupt treatment, and omission of doses must be guarded against.

Spicer & Blitz (1948) noted that, while penicillin had a bactericidal action, some cells were capable of prolonged survival and that these survivors were easily eliminated by streptomycin. Hunter (1950) and Jawetz, Gunnison & Coleman (1950) extended these observations to the enterococcus. Hunter & Paterson (1956) confirmed the superior bactericidal power of penicillin and streptomycin in combination in eliminating viridans organisms. This encouraged an attempt to shorten treatment, and reports have since appeared of two-week courses.

Hunter & Paterson (1956) collected 146 cases from 23 sources and found that only eight patients had relapsed.

Geraci (1958) reports a study with a standard treatment of two weeks of procaine penicillin (1 mega unit twice daily), probenecid (0.5 g. four times a day), and streptomycin–dihydrostreptomycin (1 g. twice daily). Of 82 patients, 16 died within nine weeks of the end of treatment; 66 were cured, but of these one certainly, and two probably, relapsed. Hall *et al.* (1955) report 15 patients, of whom five were treated for only ten days; three were not cured, but in only one was this certainly due to inadequate treatment. Less satisfactory results were obtained by Tompsett, Robbins & Berntsen (1958) in 35 cases, with relapse by three patients, one of whom relapsed twice and was finally cured by penicillin alone.

From such series it can be seen:

i. Short-term (two weeks') treatment with penicillin and streptomycin combined can yield as high a cure rate as a longer course of penicillin alone.

ii. Relapse may occur, and will usually be because of inadequate length of treatment. Finland (1958) therefore suggests that short-term treatment should be limited for organisms sensitive to 0.1 unit penicillin/ml. or less, and states that ". . . treatment . . . should be aimed at the optimum results in the greatest number of cases, despite the increased cost and discomfort to many".

iii. A risk is run of ototoxicity, although this is uncommon with the doses used.

Less sensitive strains of *Str. viridans*—those inhibited by $\geqslant$ 0.2 unit penicillin/ml.—are safest treated as *Str. faecalis*, next to be discussed.

2. *Streptococcus faecalis*

On simple plate tests, concentrations of penicillin that are high yet attainable in serum (e.g., 5–10 units/ml.) might appear adequate to cure this infection, but clinical experience demonstrated the reverse: a drug, otherwise regarded as bactericidal, was not capable of sterilizing blood or medium. Hunter (1946) showed the potency of penicillin and streptomycin in combination, and cures of enterococcal infection were reported (Cates, Christie & Garrod, 1951; Robbins & Tompsett, 1951). In the light of experience, penicillin (10–20 mega units daily), given in divided doses 3–4-hourly or by indwelling cannula, combined with streptomycin (2 g.) for at least six weeks, became basic treatment. Probenecid (0.5 g. four times a day) might be added.

Geraci (1958), guided by the bactericidal power of the treated patient's serum, has at times found it necessary to increase the daily dose of penicillin to 60 mega units, and comments that 100–150 mega units might be needed; with these levels intolerance of probenecid often occurs. If signs of ototoxicity appear and it thereby becomes desirable to reduce the streptomycin dose, he increases the dose of penicillin by 10–20 mega units, testing the efficacy of the change. He reports that with this regime the survival rate approximates to that of viridans infections.

Occasionally enterococci are encountered showing remarkable resistance to streptomycin, as in the case reported by Havard, Garrod & Waterworth (1959), in which cure was achieved by the combination of penicillin and neomycin, although the patient became deaf. Other reports of inadequacy of intensive penicillin and streptomycin treatment exist although, in some, full laboratory guidance has been lacking. A simpler programme, less of a burden to the patient, would be desirable. A solution may lie with newer antibiotics. Success has been reported with ristocetin, and vancomycin also appears promising, although both possess toxic properties. These drugs will be discussed later.

3. *Staphylococcus*

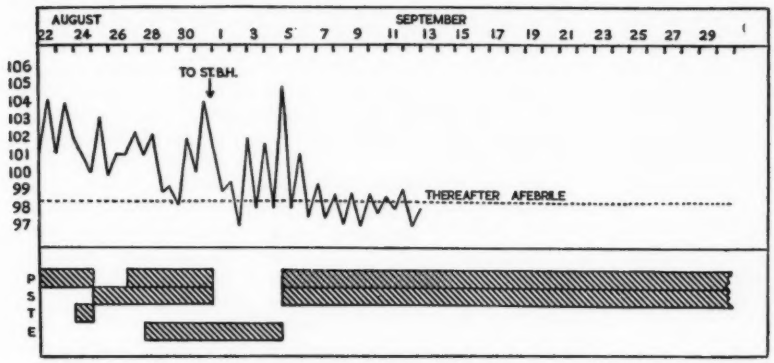
One of the most important problems of the antibiotic era has been the emergence of staphylococci resistant to an ever-increasing number of antibiotics. At the same time osteomyelitis and skin disease have tended to become less important as sources of sepsis, and staphylococcal endocarditis more commonly follows post-operative infection (Rogers, 1956). It may run an acute or chronic course and previous heart damage is present in over 50% of cases (Meade, 1959). The difficulty of treatment is reflected in mortality statistics. In some series initial improvement in figures following the introduction of penicillin has been followed by deterioration —70% mortality being reported. But this does not reveal the true state of affairs in that recent reports more often concern patients already ill or who are older. Treatment should be prolonged for at least six weeks and, because of the tendency of disseminated lesions to abscess formation, surgical intervention may be necessary. If a bactericidal drug programme cannot be realized, the most likely combination of drugs should be used.

Staphylococci differ greatly in their susceptibility to antibiotics, much depending on local prescribing habits (Barber, Csillag & Medway, 1958). Strains are frequently not homogeneous and tests must be made with an adequate inoculum (Garrod, 1955; Jawetz, Gunnison, Coleman & Kempe, 1955). From the appearance of primary plate culture, penicillinase-forming organisms can be differentiated from sensitive strains (Waterworth, 1948). The latter can be treated by penicillin alone, up to 12 mega units per day being given in divided doses, with probenecid, to delay excretion, possibly added. Combination with streptomycin may enhance the killing effect, allowing use of penicillin (4–6 mega units), probenecid (2 g.) and streptomycin (2 g.) daily in divided doses.

Statements of penicillin-sensitivity, determined by tube-dilution methods, for strains forming penicillinase, are largely meaningless, the result being dependent on the size of the inoculum (Garrod, 1958). Many of these resistant infections respond to the combination of penicillin and streptomycin. Figure 1 illustrates the value of this regime in curing a resistant staphylococcal infection which had failed to respond to initial erratic treatment elsewhere. The apparent lack of response to penicillin and streptomycin, which were in fact given in larger doses than those employed later, during the four days when erythromycin was given in addition, is of interest. The penicillin dose must be large: 10–20 mega units per day is often adequate, although 30–50 mega units have been advocated and 100 mega units or more sometimes used (Geraci, 1958). The largest doses, usually given through indwelling intravenous or intramuscular cannulae, bring their own serious problems—of electrolyte disturbances (10 mega units contain approximately 0.4 g. of sodium or 0.6 g. of potassium), yeast invasion (Jawetz, 1959) and thrombotic incidents (Moncrief, 1958).

There is, however, some debate on the part penicillin can play in very resistant infections. Finland (1958) finds it valueless; Fisher, Wagner & Ross (1953) support its use in

FIG. 1. Successful Treatment of *Staphylococcus Pyogenes* Infection of a Patent Ductus Arteriosus with Penicillin and Streptomycin



Abscissae: time (days)
Ordinates: temperature (°F.)*

P: penicillin
S: streptomycin
T: tetracycline
E: erythromycin

Female patient aged 19 was admitted to St Bartholomew's Hospital (St. B.H. in fig. 1) after treatment elsewhere. The staphylococcus was resistant to penicillin, but sensitive to other antibiotics. Tests of combined bactericidal action showed that only penicillin plus streptomycin was completely bactericidal. Penicillin (10 mega units) and streptomycin and dihydrostreptomycin (1 g. each) were given daily in divided doses for three weeks, followed by three weeks of the same dose of penicillin and half the previous dose of streptomycins. Recovery was complete and the patent ductus was later ligated.

* t° F. = § (t−32)° C.—Ed.

combination despite marked resistance on inhibition tests. Jawetz (1959) quotes such a case in which penicillin was an essential part of the antibiotic combination producing cure.

Other drugs may also show antistaphylococcal action. Bacitracin (B) and neomycin (N) are potent but have severe toxic effects, although their use to effect cure is reported. Other drugs include the tetracycline group (T), chloramphenicol (C), oleandomycin (O), novobiocin (No), streptomycin (S) and erythromycin (E). Used alone they are rarely capable of sterilizing the medium, and resistance is particularly apt to develop with No, S and E. Cures with such drugs used separately are reported, although in assessing such reports it must be recalled that endocarditis does not exist in all cases of staphylococcal bacteriaemia with heart murmur. Because of their inadequacy used singly, these drugs have been used in combination to obtain summation or, more rarely, enhancing effect, and to prevent emergence of resistance. Combinations advocated have been E with B, S, C or T; No with B, S, T or possibly C; or C with B and/or S—maximum tolerated doses being used. Melton & Logue (1957) think that the addition of B and/or E to the penicillin–streptomycin combination already discussed may be useful. Jawetz (1959), dissatisfied with the results of treatment of drug-resistant staphylococci, decided to use never less than three drugs in combination. To help choose the drug programme he tested ten drugs alone and in 19 combinations for inhibition and bactericidal power. He reports four successfully treated cases, using the following combinations: P, B, C, No and T; T, B and S; P, B, E and vancomycin; and B, E, C and P respectively. Cost apart, steering a way through such treatment was not easy despite laboratory aid, and in two of these four cases variation of the programme first proposed was necessary.

Newer drugs, vancomycin and ristocetin, are changing the pattern of treatment of resistant infections, and from preliminary reports may represent a considerable improvement.

4. Miscellaneous Organisms

Gram-negative endocarditis is infrequent, so that there is little accumulated experience. Reviews have been published for some organisms—brucella (Hudson, 1957), gonococci (Davis & Romansky, 1956) and pseudomonas, which most commonly follows surgery (Curtin, Petersdorf & Bennett, 1957). Heroic treatment is justified, for cure is difficult in the absence of potent bactericidal agents. Polymyxin, possibly because of its poor diffusing qualities, may disappoint. Suggested treatment programmes for these and other organisms are:

Brucella infections: tetracycline with streptomycin
Haemophilus influenzae infections: streptomycin with chloramphenicol or tetracycline; penicillin with streptomycin
Salmonella infections: chloramphenicol with tetracycline and/or polymyxin
Proteus infections: penicillin with chloramphenicol
Pseudomonas infections: polymyxin with possibly tetracycline and streptomycin; neomycin
Escherichia coli infections: streptomycin and tetracycline
Gonococcal infections: penicillin

5. Value of Newer Antibiotics

a. Ristocetin

Ristocetin is an antibiotic, active against gram-positive organisms, including staphylococci, and is given by vein. Initial studies showing no development of resistance or cross-resistance, and bactericidal effect at concentrations similar to those producing bacteriostasis, indicated its potential value in the treatment of staphylococcal, *Str. viridans* and *Str. faecalis*, and bacteriologically negative infections (Romansky, Limson & Hawkins, 1957). By contrast both Geraci (1958) and Schneierson, Amsterdam & Bryer (1958) report considerable difference in bactericidal and inhibition levels for strains of *Str. faecalis* and *Str. viridans*.

Weber (1958) cured two patients with resistant staphylococcal endocarditis, using ristocetin and erythromycin in combination. Gangarosa, Landerman, Rosch & Herndon (1958) cured three patients infected with *Str. faecalis*, with total doses of 22.5, 23.5, and 72.0 g. respectively. Romansky & Holmes (1958) treated successfully one staphylococcal and six *Str. faecalis* infections, five of which had failed to respond to previous treatment, and go on to mention success with *Str.*

viridans and bacteriologically negative infections. Total dosage ranged from 24 g. to 70 g. delivered over 10–24 days, with daily range of 1.5–6.0 g. With doses of 1 g. twice daily, serum levels were from 40 μg./ml. to 320 μg./ml. They advise 25 mg./kg. in divided doses 12-hourly, given in 50–100 ml. of 5% dextrose–saline over 15 minutes. Because of delayed excretion Gangarosa *et al.* (1958) recommend a loading dose of 50 mg./kg. and thereafter 10–20 mg./kg./day. Terry (1959) suggests that this drug is the best treatment for staphylococcal endocarditis, but Rantz & Jawetz (1958) mention three failures with such infection and indicate difficulty in obtaining consistent results in laboratory tests.

The incidence of toxicity—neutropenia, thrombophlebitis, fever, rashes and thrombocytopenia—lies from 9% with doses of 1.0–1.9 g. to 41% with 4.0–4.9 g. per day respectively (Herting, Lees, Zimmermann & Berryman, 1959), but purer products might cause less trouble.

b. Vancomycin

Vancomycin, active against gram-positive organisms, shows bactericidal action. Naturally resistant staphylococci are rare, and acquired resistance and cross-resistance are unimportant (Garrod & Waterworth, 1956). It must be given by vein.

Initial reports are promising. Koenig (1957) controlled a *Str. viridans* infection. Staphylococcal cures are mentioned by Schneierson, Komrad & Bryer (1958) who used the drug in combination with erythromycin, and by Geraci, Heilmann, Nichols & Wellman (1958) with vancomycin alone, in five of six patients. Bactericidal and static levels were similar, varying from 0.78 μg./ml. to 3.12 μg./ml. Treatment with 2 g. per day was of 2–4 weeks' duration, and mostly gave serum levels from 7.6 μg./ml. to 20.0 μg./ml. But levels of 80–100 μg./ml., found in two patients (only one of whom had a raised blood urea), resulted in deafness which progressed after treatment had ended. The drug tends to accumulate and, where renal impairment is present, determination of serum levels may be necessary to limit the risk (Dutton & Elmes, 1959).

6. The Bacteriologically Negative Case

From time to time patients are seen in whom the diagnosis of bacterial endocarditis may be considered a possibility, but in whom clinical certainty is lacking and blood cultures are negative. Failing a definite diagnosis such patients must be treated for bacterial endocarditis. More frequently, cases arise in which the diagnosis is unproved yet undoubted. In series reviewed by Kerr (1955) such cases formed from 10% to 33%.

While blood-culture technique may be at fault, cases tend to run to type and most positive cultures are obtained on the first, and only occasionally after the third, attempt. Of 140 positive cultures, Griffith & Levinson (1949) obtained 129 on the 1st day; 8 on the 2nd; and only 3 from later cultures: while Belli & Waisbren (1956) found 77 with the first five cultures out of 82.

The liver, and to a lesser extent lungs and deep tissues, act as bacterial filters, so that arterial bacterial counts are higher than venous, but the difference between arterial blood and that from the antecubital vein is negligible (Beeson, Brannon & Warren, 1945). Investigations into the optimum time for taking blood cultures disclosed that in any one case the count was surprisingly constant, although bacterial showers might exist; temperature changes tended to follow the rise and fall in bacteriaemia (Bennett & Beeson, 1954). Bacteria may be in a resting stage, or high levels of circulating antibodies may be present. While anti-inflammatory steroids might be expected to assist in these circumstances, their use is fraught with the risk of dissemination of untreated disease. Antibiotics, inadequate to cure, may temporarily control or reduce the degree of bacteriaemia. Organisms then recovered may show impaired growth, and growth may appear in cultures taken several days after ceasing the administration of antibiotics before it appears in those taken earlier.

Where routine blood cultures are persistently negative, and particularly where there has been previous liberal use of antibiotics, mycotic endocarditis may be present and be shown by fungal culture of blood, supported by cultures from throat, sputum and urine, and by biopsy of suitable material (Merchant, Louria, Geisler, Edgcomb & Utz, 1958). Evans, Powell & Burrell (1959) suggested that among the bacteriologically negative cases may be found examples of rickettsia endocarditis, and report a case in which at autopsy no bacteria could be demonstrated in the vegetations, rickettsia being shown by special staining.

Occasionally the history may suggest the presence of a particular organism, as when infection follows tooth extraction without prophylaxis; an appropriate treatment schedule may then be tried. Otherwise treatment should be that of a resistant organism, with large doses of antibiotics. Clinical response, reviewed early, will indicate need for increase of dose or change of drug programme.

7. Failure of Treatment

Lack of response may be apparent rather than real, for fever may accompany embolic episodes, be a manifestation of drug hypersensitivity, or be part of inflammatory reaction at the site of injected antibiotics. This sterile reaction may be more severe when massive intramuscular injections of penicillin are given. Secondary infection can occur and Jawetz (1959) mentions localized yeast abscesses in two of five patients to whom large doses of penicillin were given through intramuscular cannulae. The presence of intercurrent infection can mislead, and the differentiation between active rheumatism and bacterial endocarditis may be difficult (Horder, 1909), as is the decision regarding cure of the latter when both diagnoses are present. Signs of kidney damage often persist after infection has been controlled, and the erythrocyte sedimentation rate may not return to normal until after treatment has ended. Splinter haemorrhages may continue to be seen; they occur not infrequently in cases of uninfected mitral stenosis and can be seen in normal people (Platts & Greaves, 1958). Sweats may continue, especially in patients with aortic stenosis.

True relapse, or failure to respond to well-chosen drugs, involves other considerations. The blood-culture report may not be accurate as regards the organism or its sensitivities. It is particularly important that *Str. faecalis* and *Str. viridans* be differentiated. Reported presence of "non-pathogenic bacteria" must be seriously considered and confirmation sought in repetition of culture or detection of appropriate antibodies. In such circumstances may be found bacteria rarely reported as causing disease, e.g., *Chromobacterium prodigiosum* (Hawe & Hughes, 1954), while others are more commonly seen, Smith, Beals, Kingsbury & Hasenclever (1958) collecting over 90 cases of *Staphylococcus albus*

infection. Double infections are recorded and yeast endocarditis has accompanied treatment of bacterial endocarditis (Geiger, Wenner, Axilrod & Durlacher, 1946).

With *Str. viridans* it may usually be assumed that drug requirements for stasis closely correspond to bactericidal needs, but Berntsen (1955) has shown with certain strains that this does not apply. Other strains, e.g., *Streptococcus sanguis*, may produce high polymer sugars which possibly interfere with inflammatory repair. The difficulty of eradicating such infection was demonstrated by Robbins & Tompsett (1950) in reporting the eventual cure of such a patient after eight relapses over two-and-a-half years, and by Mikata (1958) from study of experimental infections.

Initial inadequate treatment may result in bacteria, especially staphylococci, showing increased resistance. At times, by reason of abnormal absorption and/or excretion, unexpectedly low and inadequate serum levels of antibiotics may be present, as in cases reported by Mouquin (1957) and Santos-Buch *et al.* (1957). Geraci (1958) and Dormer (1958), with illustrative cases, show the importance of a persistent focus of infection (even a buried root in an apparently edentulous person). Such foci should be sought and eradicated early in treatment.

Finally, treatment may not have lasted long enough.

8. Conclusion

To cure bacterial endocarditis we must plan to eliminate all organisms, and for this we need a bactericidal drug programme. In tests the medium must be completely sterilized, as shown by the case reported by Havard *et al.* (1959). Some antibiotics are primarily bactericidal, acting usually on actively dividing organisms; others are bacteriostatic. In combination antibiotic action may be additive, may rarely interfere, or may be potentiated (Jawetz & Gunnison, 1952; Garrod, 1953). These effects cannot necessarily be inferred, but tests can guide. With certain organisms treatment can reasonably be based on knowledge of the inhibition sensitivity tests, but it is on a firmer basis if the pattern of bactericidal action is established. For staphylococci this is essential, and in this way organism strains showing unusual characteristics will not be missed. The method of Martin, Sureau & Chabbert (1952) provides sufficient information concerning bactericidal power by a relatively simple method, but in rare instances more accurate survival counts are necessary to choose the best treatment. An adequate inoculum must be used.

The assay of bactericidal power—using a tube-dilution method—of the treated patient's serum against the infecting organism provides a check on the adequacy of treatment. Geraci (1958) uses it in all cases and has found in practice that a bactericidal effect in at least 1 in 4 dilution is satisfactory.

It is vital therefore that diagnosis shall be early, and treatment a deliberately chosen and well-planned campaign whose course should not be changed haphazardly. Treatment should be started once a thorough attempt has been made to recover the infecting organism, and should consist of penicillin in moderate doses combined with streptomycin. Modifications can be made on receipt of the laboratory report, in accordance with the principles outlined. Penicillin continues as the mainstay of treatment and should not necessarily be abandoned if hypersensitivity reactions appear. This problem may be solved by change to a different penicillin (e.g., O for G), by antihistamines (Balme & Dormer, 1954; Robinson & Binns, 1954), or by cortisone.

Penicillin and streptomycin have proved a most potent combination against a variety of organisms, and allow shorter treatment for *Str. viridans* infections, although we do not yet know the minimum duration to guarantee against relapse. Some risk of toxicity is introduced by this combination and by the newer antibiotics. The latter can be of considerable value in resistant infections but their final place cannot be assessed at present.

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THE DANGERS OF ANTIBIOTIC TREATMENT

D. M. DUNLOP B.A. M.D. F.R.C.P. F.R.C.P.E.

Professor of Therapeutics and Clinical Medicine
University of Edinburgh

Senior Physician, Royal Infirmary, Edinburgh

J. McC. MURDOCH F.R.F.P.S. M.R.C.P.E.

Senior Medical Registrar, Royal Infirmary, Edinburgh

1 Penicillin
2 Streptomycin
3 Chloramphenicol
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5 Erythromycin
6 Oleandomycin and spiramycin
7 Neomycin
8 Bacitracin
9 Polymyxin
10 Cycloserine
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12 Novobiocin
13 Antibiotic combinations
References

It is well to recall at the beginning of this brief outline of the dangers of antibiotic therapy that infectious disease has existed since man's earliest history and that his survival despite pandemics and epidemics is proof of his own inherent resistance to infection. Further, the mortality from epidemics varies at different periods of history: for example, the mortality from the pandemic of tuberculosis was falling for a very long time in Europe and North America before the advent of antituberculosis chemotherapy precipitated the decline. Moreover, there is a natural biological variation in the virulence of infecting micro-organisms, and the prevention of many infections by vaccines, antitoxins, antisera and public health measures had achieved notable successes before the antibiotic era began.

There cannot be a single branch of medicine today upon which the antibiotics do not have their repercussions, but some difficult and perplexing problems have followed in the wake of these great discoveries. The most obvious and pressing of these is the development of bacterial resistance, which is discussed in detail elsewhere in this number of the Bulletin.[1] Antibiotic therapy of necessity disturbs the patient's delicate biological balance in nature and is apt to create a bacterial vacuum which, as nature abhors a vacuum, must be filled by other micro-organisms not infrequently more dangerous than the originals; this is especially true of the so-called "hospital staphylococcus". Cross-infection in hospital wards by this organism already presents a very serious problem (Ministry of Health, 1959; *Lancet*, 1959; *British Medical Journal*, 1959). Fortunately, resistant staphylococcal infection outside hospital is still comparatively rare (Gould & Cruikshank, 1957).

The main danger in the prophylactic use of antibiotics is the creation of a false sense of security in the mind of the administrator, which may lead to the neglect of established aseptic and antiseptic techniques (*Lancet*, 1955; Blowers, Mason, Wallace & Walton, 1955; Garrod, 1955), and to carelessness in nursing methods: the concept of barrier nursing in many general hospitals has indeed been largely forgotten (Petersdorf, Curtin, Hoeprich, Peeler & Bennett, 1957; McCabe, 1959; *Scottish Medical Journal*, 1959).

[1] See Lowbury, p. 73 of this number of the Bulletin.—ED.

1. Penicillin

Of all the antibiotics in common clinical use, penicillin has been shown to produce the highest incidence of severe and fatal anaphylactoid reactions (Kern & Wimberley, 1953). Most of these reactions occur when the drug is given intramuscularly, especially in patients with a history of previous allergies. They may also occur with oral preparations (Lang & Claggett, 1955; Peters, Henderson & Prickman, 1955) and, with their more wide-spread use, such reactions may be expected to increase in the future. An estimate of all reactions to penicillin in the United Kingdom has reckoned their incidence as between 2% and 8% of all those receiving the drug (*Lancet*, 1954). The mortality rate from severe reactions, according to different surveys, has been assessed at between 9% and 13% (Welch, Lewis, Kerlan & Putnam, 1953; Welch, Lewis, Weinstein & Boeckman, 1958a). It is therefore of the greatest importance that a careful history should be taken of previous allergic phenomena before this drug is administered by any route (Calvert & Smith, 1955); and, when such a history exists, alternative antibiotic therapy should be employed. The skin reactions to the drug, of which urticaria is the commonest, vary from a mild erythema to a severe exfoliative dermatitis. Less commonly the drug may cause arthralgias resembling those encountered in serum sickness. The Jarisch-Herxheimer reaction occurs most frequently in syphilitic patients receiving intramuscular penicillin, 58% of whom experience it to a greater or less extent, the most severe and possibly fatal examples tending to arise in the tertiary stage of the disease (Scott, Maxwell & Skinner, 1949).

When penicillin is given by mouth, gastrointestinal side-effects may occur, and they include diarrhoea, heartburn, nausea and the orogenital syndrome due to the overgrowth of *Candida albicans* (*Monilia albicans*).

On the rare occasions when the use of intrathecal penicillin is indicated, no more than 5,000 units should be administered well diluted, since higher doses may lead to severe meningeal and encephalitic reactions. Patients treated with topical penicillin preparations are also liable to skin sensitization reactions; as a rule these reactions arise after about 2–3 weeks' continuous contact with the drug (Goldman, Friend & Mason, 1946). Incidentally, those concerned with the handling of penicillin tend to become carriers in the nasopharynx of strains of penicillin-resistant *Staphylococcus aureus* (Gould, 1958).

It has been suggested that there may be a causal relationship between the increased incidence of such diseases as disseminated lupus erythematosus and polyarteritis and the widespread use of penicillin during the past 15 years, but proof of this is lacking (Symmers, 1958).

2. Streptomycin

Streptomycin is capable of producing virtually all the side-effects which may result from the use of the other antibiotics. Following its administration, allergic reactions and neurotoxic, haemopoietic, gastrointestinal and renal side-effects have all been reported. Reactive symptoms have been shown to increase in severity as a result of exercise following intramuscular injection (Riches, 1954).

The early impure batches of streptomycin were capable of producing histamine-like reactions such as flushings, nausea, headache and hypotension, but these are rarely, if ever, seen with the modern purified preparations. Pyrogenic substances have also been eliminated. Local pain at the site of injection was common when the hydrochloride of streptomycin was employed, but the sulphate which is now universally used is practically painless if the injection is properly given (Thomas & Jones, 1955).

The local application of streptomycin has never been encouraged in Britain, and febrile allergic reactions from this cause are therefore encountered less frequently than with the more commonly used topical preparations of other antibiotics. Skin reactions due to parenteral streptomycin do occur, however, and generally begin with erythema going on to blistering eruptions. Such symptoms arise usually during the first three weeks of treatment and may progress to severe exfoliative dermatitis in exceptional cases (Murdoch, 1955). Contact dermatitis in those handling the drug, especially nurses, does not as a rule arise until several months of contact have elapsed. In a London hospital four of eighty nurses developed dermatitis during an 18 months' observation period (Crofton & Foreman, 1948). Rarely the mucous membranes of the mouth, pharynx and larynx may be affected by streptomycin hypersensitivity, causing grave illness and death in two reported cases (Pallister, 1949).

Damage to both the cochlear and vestibular components of the acoustic nerve by streptomycin and dihydrostreptomycin arises usually in direct proportion to the frequency and duration of their administration. The former exerts its maximal effect on the auditory portion and the latter on the vestibular portion of the eighth nerve. It is important to reduce the risk of ototoxicity in subjects over 50 years of age by giving only 1 g. thrice weekly in combination with *p*-aminosalicylic acid and isoniazid. It has also been suggested that a mixture of equal quantities of streptomycin and dihydrostreptomycin will halve the total toxicity without reducing the antibacterial activity (Sita-Lumsden & Powell, 1953). Some doubt has more recently been expressed on this assertion (Wier, Storey, Curry & Schless, 1956). Impaired renal function will lead to higher blood levels of these antibiotics and thus to a greatly increased risk of ototoxicity with even short courses of treatment (Carr, Brown, Hodgson & Heilman, 1950).

As the result of combined isoniazid and streptomycin therapy in tuberculous meningitis there is now no necessity for the prolonged daily intrathecal use of streptomycin (*Lancet*, 1957c). Thus the complete deafness and, in some cases, blindness which were occasionally distressing complications of such therapy are now fortunately extremely rare. The occurrence of central scotomata not amounting to total blindness may follow the prolonged intramuscular administration of streptomycin; but their incidence is impossible to assess without further study (Weinstein & Ehrenkranz, 1958).

Less commonly such neurotoxic phenomena as encephalopathies (Hunnecutt, Graf, Hamburger, Ferris & Sheinker, 1948), peripheral neuropathies and various psychotic disturbances have been reported as attributable to streptomycin therapy, but an exact causal relationship is doubtful.

An eosinophilia ranging from 20% to 30% is the most commonly reported blood change resulting from streptomycin therapy for tuberculosis (Farrington, Hull-Smith, Bunn & McDermott, 1947). Bone-marrow depression varies in severity but may occasionally give rise to agranulocytosis and even more rarely to aplastic anaemia and thrombocytopenic purpura (Field, 1949; Deyke & Wallace, 1948).

When given orally, streptomycin may cause mild diarrhoea due to alteration in the normal flora of the bowel, but this is seldom sufficiently severe to necessitate the cessation of treatment. A fatal case of staphylococcal enterocolitis has, however, resulted from this treatment (Kramer, 1948). French workers (Mattei, Tristani, Battei & Geraud, 1948) have reported liver damage following parenteral streptomycin, but it may well be that some of the cases were due to hepatitis transmitted by a syringe.

Casts, red cells and proteinuria caused by streptomycin therapy in the absence of previous renal disease were at one time commonly observed. So-called streptomycin nephritis is, however, much less common today following the exclusive use of the purified preparations of streptomycin sulphate.

The incidence of streptomycin-resistant staphylococci in various British hospitals now approximates to 50% (Gould, personal communication, 1959; Goodier & Parry, 1959). It has been shown that 2.3% of all newly discovered tuberculous patients in the United Kingdom are excreting streptomycin-resistant tubercle bacilli (Fox, Wiener, Mitchison, Selkon & Sutherland, 1957). These are capable of transmission from person to person (Thomas, Borthwick, Horne & Crofton, 1954), and apparently show no loss of virulence for man as a result of their resistance to the antibiotic.

It will be appreciated, therefore, that the use of streptomycin alone in the treatment of tuberculous and staphylococcal infections is to be avoided at all costs. Probably it would be wiser at the present time to avoid streptomycin therapy, except in combined antituberculosis chemotherapy, until it is known that the organisms causing the infection are fully sensitive to its action.

3. Chloramphenicol

A variety of gastrointestinal reactions to this antibiotic have been recorded, such as nausea, glossitis, stomatitis, diarrhoea and pruritus ani. Such reactions are commoner following tetracycline therapy and will be discussed more fully under that heading. Chloramphenicol is less destructive to the intestinal flora than the tetracyclines, and the low incidence of diarrhoea caused by it may be due to its rapid absorption from the jejunum and the prompt inactivation of what remains in the intestine (Woodward & Wisseman, 1958).

Allergic skin reactions such as occur more commonly following the use of penicillin have been reported in patients sensitive to chloramphenicol. These usually consist of angioneurotic oedema or vesicular and maculo-papular types of dermatitis. These dermal lesions as a rule subside rapidly when the administration of the antibiotic is stopped.

The Jarisch-Herxheimer reaction, first noted as a sequel to the arsenical and then to the penicillin treatment of syphilis,

has been reported following treatment by chloramphenicol of patients suffering from syphilis, brucellosis and typhoid fever (Romansky, Olansky, Taggart & Robin, 1949; Knight, 1950; Galpine, 1949; Marmion, 1952). This reaction, characterized by a greater or less degree of circulatory collapse, has been ascribed to the sudden release of endotoxin from killed organisms, but the true explanation is still in doubt.

Neurotoxic manifestations occurring during the course of prolonged treatment with chloramphenicol have been very rare and not unduly troublesome, but vertigo, convulsions, optic neuritis and digital paraesthesiae thought to be due to a toxic polyneuritis have been reported (Wallenstein & Snyder, 1952). It is possible, however, that such toxic phenomena may have been due to the disease for which chloramphenicol was being administered rather than to the drug itself.

Soon after the initial clinical trials with chloramphenicol were undertaken in 1948, it became apparent that it was well tolerated and, because of its apparent comparative freedom from undesirable side-effects, the antibiotic began to be used in a very free and irresponsible manner. Reports on its adverse haematological effect first appeared in the medical literature in 1950 and it was concluded that on rare occasions in susceptible individuals, particularly when the drug was given in intermittent courses, or in prolonged and excessive doses, it might cause various blood dyscrasias, especially aplastic anaemia but also including thrombocytopenic purpura and agranulocytopenia (Rich, Ritterhof & Hoffman, 1950; Gill, 1950). It is only fair to point out that in many of the reported instances the blood changes attributed to chloramphenicol might equally well have been due to other drugs which the patient was receiving at the same time (Hodgkinson, 1954; Welch, Lewis & Kerlan, 1954). It is known that by 1954 over 8,000,000 patients had received chloramphenicol, and the incidence of blood dyscrasias due to it must be viewed against this background (Wayne, 1958). The total number clearly attributable to its administration was of the order of a few hundreds, giving an incidence of between 1 in 10,000 and 1 in 100,000. Perhaps the former higher incidence is likely to be the more correct, since by no means every case is recognized or recorded. While, therefore, the association of many cases of aplastic anaemia and other blood dyscrasias attributed to chloramphenicol may have been coincidental, it is right that this potential hazard should be kept in mind in prescribing it (Garrod, 1958). In general, the dosage should be limited to 2 g. a day for five days, except when chloramphenicol is the only possible treatment as in typhoid fever. When the total course is limited to 10 g., and not more than one course is given, the risk of causing blood dyscrasias is minimal (Ioannidis & Murdoch, 1957).

4. Tetracyclines

The allergic and neurotoxic reactions, nausea and blood dyscrasias which have already been described as side-effects to other antibiotics are very rarely encountered when the tetracyclines are given. The main dangers in this form of treatment concern the overgrowth of two potentially pathogenic micro-organisms resistant to the tetracyclines in the gastrointestinal, respiratory and genito-urinary tracts. One of these—*C. albicans*—is common to all patients whether treated at home or in hospital; the other—*Staph. aureus*—is at present largely confined to the hospital environment.

With the marked depression by tetracycline treatment of the normal bacterial flora inhabiting the skin, nasopharynx and alimentary and urogenital tracts, an inevitable overgrowth to a greater or less extent occurs in the population of *C. albicans* inhabiting these regions. A mild overgrowth may lead to hairy tongue, glossitis, stomatitis and pruritus ani and vulvae (Anderson, 1956; Ronald, 1956; Stokes, 1956). At one time it was considered that such syndromes were due to the impairment of vitamin B production as the result of destruction in the intestine of the coliform organisms responsible for its synthesis. Since these syndromes might occur within forty-eight hours of starting tetracycline treatment, and since they were unrelieved by vitamin B complex therapy, this explanation has been largely discarded. Further, tuberculous patients receiving as much as 4 g. or 5 g. of oxytetracycline a day for many months showed no evidence of avitaminosis (Stewart, Turnbull & Crofton, 1954; Murdoch & Stewart, 1956).

Less commonly, but much more seriously, a more profuse overgrowth of *C. albicans* may reach invasive proportions leading to serious involvement of the mucosa of the alimentary, respiratory and genito-urinary tracts that may even progress to a fatal monilial septicaemia. Such invasions lead to sloughing and ulcerative lesions in the regions involved, characterized clinically by high temperature, profound prostration and excessive mucopurulent discharges from the affected system. In pulmonary cases severe dyspnoea and cough occur, with the expectoration of copious stringy mucus. Intestinal involvement leads to the passage of frequent, odourless, loose, mucopurulent and, sometimes, bloody stools. In fatal cases wide-spread monilial abscesses have been found in the liver, kidneys and brain. Secondary infections due to monilia are fortunately infrequent (Welch, Lewis, Weinstein & Boeckman, 1958b).

It is now well recognized that a large proportion of the staphylococcal population in hospitals in the United Kingdom and the United States of America is resistant to the antibiotics which are commonly employed. The clinical problems of drug-resistant pathogens are discussed elsewhere in this number of the Bulletin,[2] and the problem of staphylococcal infections in British hospitals is summarized in the report of the Standing Medical Advisory Committee of the Central Health Services Council to the Ministry of Health, and this report is commented on in recent editorials in the *British Medical Journal* (1959) and *Lancet* (1959). When resistant staphylococcal superinfection arises serious and often fatal enterocolitis, characterized by a fulminating cholera-like illness, may result. This is seen most frequently post-operatively and in neonatal and paediatric wards (Gould & Allan, 1954; Shooter, Smith, Griffiths, Brown, Williams, Rippon & Jevons, 1958). In general medical wards this organism more commonly invades the respiratory and urinary tracts of patients receiving tetracycline treatment, giving rise to pneumonia, which may progress to multiple lung abscesses, or to a necrotizing cystitis. Following long-term tetracycline therapy in selected cases of chronic bronchitis, a high percentage of such patients has been found to harbour resistant staphylococci in the anterior nares; and such organisms are presumably potentially pathogenic and may invade the susceptible bronchial mucosa (May & Oswald, 1956). This has been seen particularly during epidemics with the influenza virus when secondary staphylococcal pneumonia has frequently contributed to a fatal outcome (Walker, Douglas,

[2] See Lowbury, p. 73 of this number of the Bulletin.—Ed.

Leckie, Pines & Grant, 1958). There is also a considerable danger in admitting patients with an acute exacerbation of chronic bronchitis into a general hospital ward, since once the susceptible pneumococci and *Haemophilus influenzae* responsible for the acute illness have been eliminated by antibiotics commonly employed, a superinfection by the resident and resistant staphylococci is an ever-present menace. It would seem therefore in such circumstances that antibiotic treatment by the patient's general practitioner at home, in spite of possibly inadequate nursing facilities, is often preferable to hospital admission.

The side-effects which have been mentioned are common to all three tetracyclines—oxytetracycline, chlortetracycline and tetracycline itself. Nevertheless it has been suggested that the incidence of simple diarrhoea (i.e., rather frequent soft, bulky, odourless stools, said to be due to the chemical nature of the antibiotic) varies with the antibiotic used, but proof of this is lacking. In any case the symptoms disappear rapidly when the treatment is stopped, and the use of lower doses reduces the risk of severe diarrhoea (Finland, Grigsby & Haight, 1954). *Staph. aureus* may be cultured from the stools of about 15% of patients with diarrhoea due to tetracycline therapy (Frei, Auner, van Metre & Zubrod, 1955), but they can also be demonstrated, if selective methods of cultivation are used, in the faeces of patients with no symptoms (Brodie, Kerr & Sommerville, 1956).

5. Erythromycin

When given orally this drug is responsible for mild gastrointestinal side-effects, as it is directly stimulating to intestinal motility. Thus the passage of soft stools is common even after a short course of treatment. Such side-effects are seen in 29% of patients receiving 1.8–2.2 g. of erythromycin per day (David, 1955). Nausea, vomiting and colicky abdominal pain are due to the erythromycin itself and not to the vehicle in which it is administered, as these symptoms arise whether the antibiotic is given in capsule, tablet or enteric-coated tablet form.

Allergic reactions are rare and are mainly related to topical application (Livingood, Head, Johnson & Nilasena, 1953). Side-effects in other systems have not been reported so far. On the whole, erythromycin must be judged as a remarkably safe antibiotic from the point of view of toxicity, but topical application should be avoided (Herrell, 1955). Where other antibiotics give rise to toxic symptoms, erythromycin is likely to be well tolerated (Forfar & McCabe, 1957b).

6. Oleandomycin and Spiramycin

It has been shown that these two antibiotics are generally inferior to erythromycin, thus indicating that their adoption for general use in the treatment of infections should be actively discouraged (*Lancet*, 1957b). They should be employed only where it is definitely proved that the infection is caused by organisms which are highly sensitive to these agents and resistant to erythromycin as well as to other active antibiotics in common use (Jones & Finland, 1957a). The indiscriminate use of these weak erythromycin-like antibiotics—so widely advertised to medical practitioners—may well lead to serious difficulties due to the high incidence of staphylococcal cross-resistance between erythromycin, spiramycin and oleandomycin (Garrod, 1957).

7. Neomycin

Serious side-effects may follow the parenteral administration of this antibiotic. The most notable of these is the occurrence of severe necrosis of the renal tubular epithelium. Fatal cases resulting from such therapy have been recorded (Powell & Hooker, 1956; Perry, 1952). The use of neomycin in the presence of previous or active renal disease is completely contra-indicated for this reason. Eighth nerve damage, similar to that caused by streptomycin, but more severe in that it usually arises more rapidly, is another untoward effect which limits its parenteral use (Waksman, 1953).

On the other hand, this antibiotic is well tolerated by mouth as it is poorly absorbed by the intestinal mucosa. Minor discomfort from nausea, and the passage of soft stools, may arise after prolonged oral administration.

8. Bacitracin

Early preparations of bacitracin, containing bacitracins A, B, C, and F, were quite unsuitable for parenteral administration as severe nephrotoxicity invariably resulted. It was suggested that the parenteral route be totally abandoned (Newton, Abraham, Florey, Smith & Ross, 1951). The more modern preparations with a high content of bacitracin A are now safe for systemic use if the daily intake of fluid is 2,500 ml. or more, and the urinary output exceeds 1,000 ml. (Meleney, 1956). Oral and topical therapy with this antibiotic, when indicated, are relatively safe and free from serious toxicity.

9. Polymyxin

The name polymyxin also refers to a family of antibiotics, of which the least toxic are B and particularly E. Local pain at the site of intramuscular injections is particularly severe and prolonged in susceptible adult patients (Swift & Bushby, 1953). Neurotoxicity may lead to paraesthesiae, especially around the mouth and in the "glove and stocking" distribution, and to flushings, vertigo and ataxia. These alarming symptoms disappear without sequelae within seventy-two hours of stopping treatment (Kagan, Krevsky, Milzer & Locke, 1951; Jawetz, 1952).

Nephrotoxicity is clinically manifested by the appearance of proteinuria and urinary casts and is common when polymyxin is given in parenteral doses of 2.5 mg./kg. daily. Higher doses will cause definite impairment of renal function and are contra-indicated. When previous renal disease is present or suspected, not more than 1 mg./kg. daily for 5–10 days should be given (Hopper, Jawetz & Hinman, 1953).

Allergic responses to polymyxin are rare, arising in probably less than 0.1% of patients (Jawetz, 1956).

10. Cycloserine

When given in daily oral doses of over 1 g., this antibiotic causes marked neurotoxicity. Drowsiness, psychotic reactions, myoclonus, subjective twitchings, vertigo and epileptiform seizures are frequent clinical manifestations. These effects are rapidly reversible when the drug is withdrawn. Sudden cardiac decompensation leading to death in three patients may have been contributed to by the administration of cycloserine in a total daily dose of 1.5 g. or more (Walker & Murdoch, 1957). This drug should be given, when indicated, in an initial dose of 0.25 g. twice daily and gradually increased to a total daily dose of not more than 1 g.

11. Vancomycin

This antibiotic requires to be given by the intravenous route and, if administration is repeated or prolonged, thrombophlebitis is frequently produced. This can be largely avoided by using dilute solutions and varying the sites of injection. In the presence of impaired renal function, unnecessarily high blood levels of about 100 μg./ml. may damage the eighth cranial nerve and cause deafness or tinnitus (Geraci, Heilman, Nichols & Wellman, 1958). Dutton & Elmes (1959) who treated 9 patients with vancomycin, record the development of deafness in 4 of them, 3 of whom had impaired renal function. These authors recommend smaller doses in such patients, preferably given by continuous drip to maintain a steady level, the dosage being controlled by serial rapid assays of the antibiotic content of the blood.

Drug fever and skin rashes have arisen as a result of vancomycin therapy, but anaphylactoid reactions have not so far been described.

12. Novobiocin

Since its recent introduction into therapeutics, nausea, vomiting, diarrhoea, yellow sclerae and various skin rashes have already been attributed to novobiocin (Welch, Lewis, Putnam & Randall, 1956; David & Burgner, 1956; Nichols & Finland, 1956; Martin, Heilman, Nichols, Wellman & Geraci, 1956). Much the most common and troublesome of these effects are skin rashes, usually urticarial and often severe and febrile: these occur in about 10% of all patients given full doses for more than one week. A case of agranulocytosis and one of serious thrombocytopenia have also been recorded following its administration (Colville, Gale, Cox & Quinn, 1958). More wide-spread use of this antibiotic can be expected especially in the treatment of hospital-acquired staphylococcal infections, and careful observation of the blood picture should be made in these circumstances. Rapid development of resistance to novobiocin by the *Staph. aureus* is a further factor in deciding upon its rational use (Jawetz, Bertie & Sonne, 1957).

13. Antibiotic Combinations

Combinations of antibiotics which block different metabolic processes in micro-organisms have an established place in preventing the emergence of drug-resistant variants as in the treatment of tuberculosis. Synergism between antibiotics to produce bactericidal activity greater than the additive effects of a given combination has now been claimed for several different pharmaceutical preparations. The position with regard to the danger and disadvantages of such combinations has been recently commented on in several authoritative papers and leading articles (Cruickshank, 1957; Forfar & McCabe, 1957a; Jones & Finland, 1957a, 1957b; *Lancet*, 1957a, 1957b; *New England Journal of Medicine*, 1956). They point out that proof of synergism is still lacking in clinical practice, and very recently it has been shown that staphylococcal cross-resistance between such antibiotics as erythromycin and chloramphenicol can be induced by such a combination (Barber, Csillag & Medway, 1958). New combinations of antibiotics should be thoroughly investigated both in vitro and in vivo, and submitted to cautious clinical study before being released for general use in the community.

From the foregoing it will be apparent that we have not yet achieved Ehrlich's utopian ideal of a specific agent for a specific infection without harm to the host. At present the wide-spread and often empirical prescription of antibiotics is becoming something of a menace to the population, not only because of their potentially dangerous side-effects but also because of the inherent adaptability of certain micro-organisms to an antibiotic environment.

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CLINICAL PROBLEMS OF DRUG-RESISTANT PATHOGENS

E. J. L. LOWBURY M.A. D.M.

Medical Research Council
Industrial Injuries and Burns Research Unit
Birmingham Accident Hospital

1 Staphylococcal infections
  a Resistance of *Staphylococcus aureus* to individual antibiotics
  b Types of staphylococcal hospital infection
  c Association of resistance with the use of antibiotics
2 Infection by some other bacteria
  a Streptococci
  b Gram-negative bacilli
  c *Neisseria gonorrhoeae* and *Neisseria meningitidis*
  d *Mycobacterium tuberculosis*
3 Measures to combat the emergence of resistant pathogens
4 Comments and conclusions
References

The clinician is placed in a dilemma through the emergence of pathogens resistant to the antibiotics;[1] for the more these drugs are used, the less useful they are likely to become in the treatment of certain common infections, especially those caused by the staphylococci, the gram-negative bacilli and by *Mycobacterium tuberculosis*. To the biologist this result is not surprising, for it illustrates the combined effects of variation in the bacterial species and selection by environmental factors. It is, perhaps, more surprising—and for medicine most fortunate—that resistant strains of certain other bacteria (e.g., *Treponema pallidum*, *Streptococcus pyogenes*, *Actinomyces israeli* and *Clostridium welchii*) do not emerge readily (or at all) under these conditions. Moreover, in the same bacterial species resistance emerges readily towards some drugs and rarely or not at all towards others.

Four factors—the host, the pathogen, the antibiotic and the environment—are involved in the chemotherapy of infection. Studies on habituation in vitro over-simplify the picture by ignoring two of these factors. Resistant forms appearing in culture media, for example, may have little relevance to chemotherapy, for such variants are often unable to flourish in human tissues. The emergence of resistance depends not only on the frequency of resistant variants, but also on the frequency of contact between antibiotic and organism; the latter is determined by the ecological characteristics of the organism, and also by its accessibility and response to the administration of an antibiotic.

For the clinician the term "resistance" implies, among other things, that treatment with the drug against the organism in question will be ineffective. In the laboratory this criterion is, to some extent, paralleled by describing an organism as resistant if the lowest concentration of antibiotic required to inhibit its growth is higher than that which reaches the infected tissues during treatment; this will obviously vary with the mode of administration, and an organism may, theoretically, be resistant to systemic therapy but sensitive to local therapy with the same antibiotic. In practice, somewhat arbitrary levels and a great variety of sensitivity tests are used; the literature is, in consequence, full of divergent views on the resistance of certain bacteria.

Two separate mechanisms may cause the replacement of sensitive by resistant bacteria of the same species in the tissues of a patient receiving an antibiotic: (i) the strain originally present in the lesion may become resistant; and (ii) the lesion may become secondarily colonized by resistant bacteria present in the environment. In either case the resistant strain becomes predominant in the hospital environment through selection and cross-infection.

[1] For convenience the word "antibiotic" is used here to cover all antibacterial chemotherapeutic agents.

1. Staphylococcal Infections

Hospital infection by resistant *Staphylococcus aureus* has been intensively studied in recent years (see, for example, Rountree & Thomson, 1952; Barber, 1958; Finland & Haight, 1953; Elek, 1959). The organism is well equipped for the role, as it is normally carried by about 50% of healthy adults, is commonly found on the skin, causes superficial infections, survives well in dust and has outstanding ability to produce antibiotic-resistant variants.

A large proportion (often more than 80%) of the staphylococci isolated today from patients in hospital are resistant to penicillin, and at least half as many are usually resistant also to the tetracyclines; a variable proportion are resistant to streptomycin, chloramphenicol, erythromycin, and even to novobiocin. Resistance is found most commonly in staphylococci of phage group III and of certain patterns in phage group I (Barber & Whitehead, 1949; Rountree & Thomson, 1949; Barber & Burston, 1955; Rountree & Freeman, 1955). Staphylococci carried by the general population show a much lower incidence of antibiotic-resistance, and more of the strains belong to phage group II; in towns, penicillin-resistant strains increased during the middle 1950's to about 20% (Rountree & Rheuben, 1956; Rees, Shooter & Shawe, 1955; Roodyn, 1956). The tendency for penicillin-resistant strains to accumulate varies considerably with the locality (cf. Wysham & Kirby, 1957; Griffith, Boniece, McGuire, Wolfe, Joiner, Wick & Holmes, 1958). Resistance to other antibiotics is rare outside hospitals.

a. Resistance of Staphylococcus aureus *to Individual Antibiotics*

Penicillin. In the early studies on penicillin it was shown that resistance could be induced by growing sensitive strains in subinhibitory concentrations of the antibiotic (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941), but such strains lacked virulence (Rake, McKee, Hamre & Houck, 1944) and did not produce penicillinase (Abraham *et al.* 1941; Bondi & Dietz, 1944), the penicillin-destroying enzyme which was found to be produced by virtually all resistant staphylococci from infections (Bondi & Dietz, 1945; Barber, 1947).

These findings have an important bearing on the clinical use of penicillin. As mutation to penicillinase production is very rare (Szybalski, 1953), a sensitive infecting strain is most unlikely to acquire resistance during treatment, though replacement by a resistant strain often occurs in open lesions

treated in hospital. In addition to rendering the staphylococcus resistant to penicillin, staphylococcal penicillinase may protect other pathogens (e.g., *Str. pyogenes*) when these happen to be present in the same lesion (Cruickshank, Squire & Topley, 1948; Jackson, Lowbury & Topley, 1951b; Tacking, 1953; Körlof, 1954). As the amount of penicillinase produced varies and the staphylococcal cells that produce it are sensitive, it might be thought that penicillin in adequate dosage should sometimes be effective in treatment of infection by "resistant" organisms (Bondi & Dietz, 1948; Burn, Curwen, Huntsman & Shooter, 1957). But penicillinase production is apt to increase in response to penicillin therapy (Geronimus & Cohen, 1957) and, since such staphylococci today are in most cases highly resistant to penicillin (Finland, 1955b), the chemotherapy directed against them will naturally be chosen from one or more of the other available antibiotics.

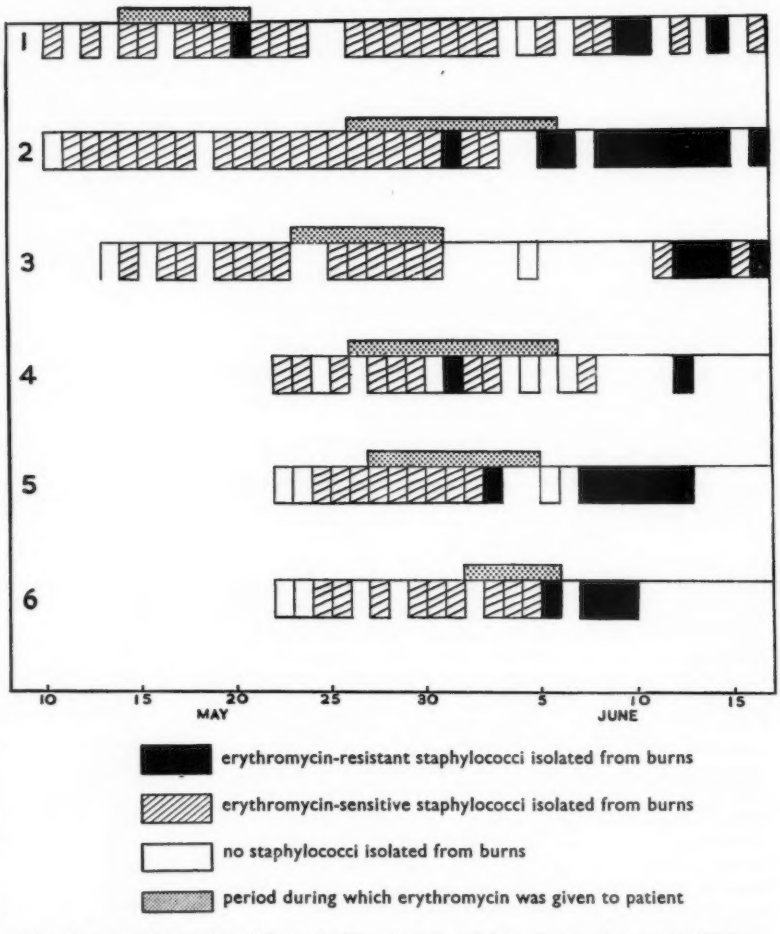
Streptomycin. Staphylococci, like other organisms, acquire resistance to streptomycin more readily than to any other antibiotic. Because of its importance in the treatment of tuberculosis, it is desirable to avoid the use of streptomycin for staphylococcal and other infections when effective alternatives are available.

The tetracyclines. Mutants resistant to the tetracyclines emerge rather rarely (Paine, Collins & Finland, 1948), but they may arise in patients receiving a tetracycline (Nichols & Needham, 1949) and show no loss of virulence. In hospitals where much tetracycline is used, tetracycline-resistant staphylococci are common (see, for example, Finland, 1955a; Lowbury, 1958). In a controlled prophylactic trial of chlortetracycline in a burns Unit, all the staphylococci were sensitive at the outset, but after six months over 50% of the staphylococci isolated in the Unit were resistant; the degree of resistance of individual strains increased in the later months of the trial (Lowbury, Topley & Hood, 1952).

Chloramphenicol. Resistance to chloramphenicol, as to the tetracyclines, emerges slowly (Barber, 1953) but becomes common in hospitals where much chloramphenicol is used (Chabbert & Terrial, 1952). In general, chloramphenicol-resistant strains of staphylococci have been relatively rare (often less than 10%) in hospitals (see, for example, Royer, 1958), presumably because of the restricted use of this antibiotic since it has been known to cause blood dyscrasias.

The erythromycin group. Staphylococci resistant to these antibiotics emerge readily and may become predominant in hospitals (Hobson, 1954; Lepper, Moulton, Dowling, Jackson & Kofman, 1953; Lowbury, 1958). During an eight months' trial of erythromycin given in small dosage usually for six days, resistant strains were not found (Lowbury & Cason, 1954). Later, however, erythromycin-resistant staphylococci of the same phage type as sensitive staphylococci previously present appeared in the burn of a patient who was having a course of erythromycin; and soon erythromycin-resistant staphylococci—not all of the same phage type—appeared in the burns of a number of other patients receiving erythromycin (see fig. 1).

FIG. 1. Appearance of the First Erythromycin-Resistant Staphylococci in a Burns Unit, among Six Patients who were Receiving Oral Erythromycin Therapy



The six patients (numbers 1–6 on the figure) were receiving oral erythromycin therapy for infection with *Streptococcus pyogenes*.

Sensitive (S) and resistant (R) *Staphylococcus aureus* isolated from burn swabs showed the following phage types:

Patient 1, S and R: 75B
Patient 2, S and R: 75B, 42B/47C/47D/53 and NT (not typable)
Patient 3, R: 75B, 53/57
Patient 4, S: 3C+, 52A+, 75B+ and NT; R: 42B/47C/47D/53/57
Patient 5, S: 75B, 55/71, NT; R: 75B, 53/70+
Patient 6, S: 53, 75B; R: 75B

A policy of restraint in the use of erythromycin has undoubtedly helped to delay the rise of resistant staphylococci in many hospitals. With the arrival of the related antibiotics oleandomycin, spiramycin and E129, new opportunities arose for the selection of staphylococci resistant to erythromycin; cross-resistance, however, is incomplete in this group, especially in strains from patients, and spiramycin and E129, in particular, may retain activity against staphylococci which have become resistant to erythromycin (Ross, 1956; Hudson, Yoshihara & Kirby, 1956; Garrod & Waterworth, 1956). The degree of cross-resistance varies with the strain of staphylococcus, one or another pattern tending to predominate in particular environments (Garrod, 1957). Most erythromycin-resistant strains which appear sensitive to oleandomycin grow in the presence of that antibiotic on continued incubation (Lowbury & Hurst, 1959).

Novobiocin. This antibiotic shares with erythromycin outstanding activity against *Staph. aureus* and exceptional ability to induce the emergence of resistant staphylococci in vitro and in vivo (Nichols & Finland, 1956; Garrod & Waterworth, 1956). Novobiocin-resistance can easily be added to the repertoire of hospital staphylococci by the treatment of burned patients with novobiocin (Lowbury, 1957).

Vancomycin. In contrast with the antibiotics discussed above, vancomycin is active against all strains of *Staph. aureus*, and significant resistance does not emerge on attempted habituation in vitro (Garrod & Waterworth, 1956). Vancomycin appears to have considerable value in the treatment of severe or generalized hospital infections with *Staph. aureus* (Dutton & Elmes, 1959; Davis, Ramsay & Mann, 1958).

Ristocetin. Like vancomycin, this new antibiotic does not appear to induce the emergence of resistant staphylococci (Fairbrother & Williams, 1958).

Bacitracin, neomycin and framycetin. Resistance to these three antibiotics develops readily in vitro, but is rarely found in vivo. During the severe test of local prophylactic trials in a burns Unit, we found no staphylococci resistant to neomycin or to bacitracin among many hundreds examined. Their toxicity precludes systemic use of these drugs—especially of the related antibiotics, neomycin and framycetin—except as a life-saving measure (see, for example, Havard, Garrod & Waterworth, 1959), but they are suitable for local application.

b. Types of Staphylococcal Hospital Infection

i. Wound infection. The predominance of antibiotic-resistant *Staph. aureus* as a cause of post-operative and open-wound infection has been reported by many workers (e.g., Shooter, Griffiths, Cook & Williams, 1957; Shooter, Smith, Griffiths, Brown, Williams, Tippon & Jevons, 1958; Lowbury, 1955). Howe (1954) and Blowers, Mason, Wallace & Walton (1955) produce evidence to suggest that such infections have actually increased with the increasing use of antibiotics, perhaps through the selection of more virulent and more communicable strains, and probably also through the widening scope of operative surgery and through deficiencies in aseptic technique.

ii. Infection in maternity departments. Many accounts of epidemic and endemic infection have been published (e.g., Barber, Hayhoe & Whitehead, 1949; Colbeck, 1949; Timbury, Wilson, Hutchison & Govan, 1958; Gillespie, Simpson & Tozer, 1958; Beavan & Burry, 1956). A high carrier rate of resistant staphylococci occurs in infants as well as staff; cross-infection from patients may be more important than from nasal carriers in the nursing staff (Forfar, Balf, Elias-Jones & Edmunds, 1953). The types of infection include breast abscesses in the mothers, and in the infants lesions of the skin and the eyes, abscesses, cellulitis and pneumonia. The staphylococci causing infection in maternity departments are often of group I, and some of the most severe outbreaks have been associated with strains of phage type 80; group III strains are also commonly found.

iii. Respiratory infections. The commonest bacterial complication in the epidemic of Asian influenza was a staphylococcal pneumonia, often due to a resistant strain, particularly when developing after some days in hospital, and sometimes fulminating (Public Health Laboratory Service, 1958; Robertson, Caley & Moore, 1958). In contrast with non-staphylococcal pneumonia, these infections had approximately the same mortality in all age-groups (Oswald, Shooter & Curwen, 1958). Severe staphylococcal pneumonia has apparently become much commoner in childhood since the arrival of broad-spectrum antibiotics (Prissick, 1953; Wallman, Godfrey & Watson, 1955); it has also been a common cause of death in debilitated hospital patients (Gresham & Gleeson-White, 1957).

iv. Intestinal superinfection during antibiotic therapy. The use of antibiotics, especially oral therapy with tetracyclines, has been responsible for a great increase in frequency of severe enterocolitis due to superinfection with antibiotic-resistant staphylococci (Fowler, 1955; Cook, Elliott, Elliott-Smith, Frisby & Gardner, 1957). Such infections are often fatal, and it is important to have available for their treatment antibiotics to which the organisms are fully sensitive.

c. Association of Resistance with the Use of Antibiotics

The proportion of resistant staphylococci in a hospital environment is closely associated with the amount of antibiotic used there. An example of this (see fig. 2) was obtained in a burns Unit where at first tetracycline and later erythromycin was the antibiotic in routine use for the treatment of streptococcal infection (Lowbury, 1958).

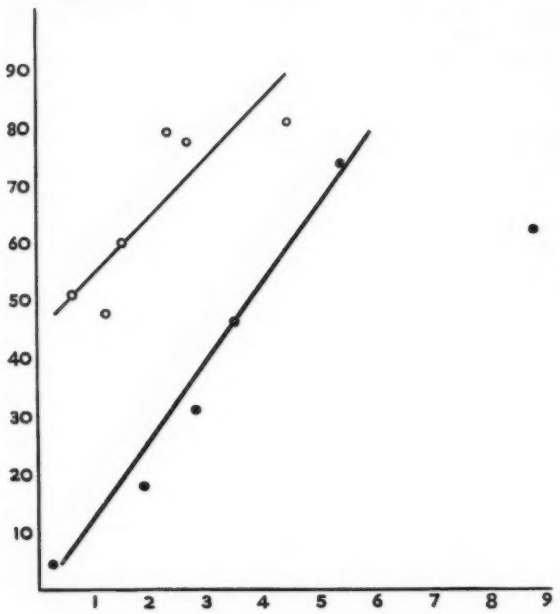
2. Infection by some other Bacteria

a. Streptococci

Chemotherapy has scored one of its greatest successes against *Str. pyogenes*, which is today of secondary importance as a cause of puerperal or wound infection. Penicillin-resistant strains have not been found (see, for example, Denny, 1954). Unfortunately, penicillin therapy is not effective when the streptococcus is accompanied by penicillinase-producing organisms (see section 1a). For such infections the tetracyclines and erythromycin are highly effective; but strains of *Str. pyogenes* resistant to the tetracyclines, as to the sulphonamides, can emerge and become predominant in environments where cross-infection is common. Infection by such strains does not respond to tetracycline therapy (Lowbury & Cason, 1954; Lowbury & Hurst, 1956; Lowbury 1958).

Strains of *Streptococcus viridans* showing increased resistance to penicillin are occasionally found in patients with relapses of subacute bacterial endocarditis after treatment (Cates & Christie, 1951), but resistance to penicillin is commoner among strains of *Streptococcus faecalis* (Robbins &

FIG. 2. Association of Resistance in *Staphylococcus Aureus* with the Use of Antibiotics in a Burns Unit



In a period of three years, the percentage of resistant strains among the first 20 staphylococci isolated each month is plotted against the average number of patients treated with the antibiotic in the last week of the previous month.

Tompsett, 1951; Cates, Christie & Garrod, 1951), and resistance to the tetracyclines and erythromycin is not uncommon (Finland, 1954). In the treatment of infection with such strains, the combined administration of penicillin with streptomycin, or with neomycin (Havard *et al.* 1959), has proved efficacious—treatment with the latter, unfortunately, sometimes complicated by deafness. Other potentially useful agents which are consistently active against *Str. faecalis* in vitro are vancomycin and ristocetin.

b. Gram-Negative Bacilli

Infection by the gram-negative bacilli has proved even more difficult to manage with antibiotics than has staphylococcal infection. Many of these organisms include a mixture of sensitive and resistant strains, and most of the antibiotics effective against the group are predominantly bacteriostatic. Even when the organisms are sensitive, it may be hard or impossible to dislodge them by antibiotic therapy.

i. Intestinal infections. Organisms of the shigella group can acquire resistance to the sulphonamides during treatment (Hardy, 1945); administration of chloramphenicol, however, has not been found to induce the emergence of resistant strains (Mazursky, 1950; Sime & Cameron, 1951). Good results in the treatment of bacillary dysentery without emergence of resistant shigellae have also been claimed for the tetracyclines (Kunstadter, Milzer & Kagan, 1951) and for neomycin (Rogers, Benson, Foster, Jones, Butler & Williams, 1956).

The salmonellae present greater difficulties. Relapses are common after initial success in the treatment of typhoid fever by chloramphenicol; the organisms remain sensitive, but are often not eliminated by the antibiotic, perhaps because it is predominantly bacteriostatic (Woodward, 1949; Rumball & Moore, 1949). Neomycin has been found to have some value in the treatment of infection by *Salmonella typhi-murium*, but sensitive salmonellae are liable to reappear after apparently successful treatment even with this bactericidal drug (Neter, 1958).

All strains of enteropathogenic *Escherichia coli* serotypes appear to be highly or moderately sensitive to neomycin, and only small or moderate increases of resistance are acquired in vitro or during treatment with that antibiotic; relapses after neomycin treatment of infantile gastroenteritis are, nevertheless, not uncommon (see Gorzynski & Neter, 1953; Rogers *et al.* 1956).

ii. Infections of the urinary tract and of wounds. The gram-negative bacilli which cause these infections include the common intestinal *E. coli*, *Pseudomonas pyocyanea*, and various species of *Proteus*, *Klebsiella*, *Citrobacter* and paracolon bacilli. The frequency and intractability of hospital infection with *Ps. pyocyanea* have been emphasized, but all strains of this organism appear to be killed by low concentrations (0.5–5.0 μg./ml.) of polymyxin (B or E) (see Jackson, Lowbury & Topley, 1951a; Sherwood, Delage & Herman, 1953). No antibiotic shows a similar uniformity of action against any of the other gram-negative bacilli involved in infection of wounds or of the urinary tract (see, for example, Pottee, Wright & Finland, 1954; Garrod, Shooter & Curwen, 1954), and the more resistant species of *Proteus*, *Klebsiella* and paracolon bacilli appear to be selected in urological or burns wards. Chloramphenicol is probably the most useful single agent for these infections, but the selection of an antibiotic or of antibiotics must be controlled by sensitivity tests. Antibiotic therapy is apt to be frustrated in these fields both by the resistance and by the inaccessibility of the infecting organism.

c. Neisseria gonorrhoeae *and* Neisseria meningitidis

Soon after the sulphonamides became widely used, sulphonamide-resistant gonorrhoea was found to be common (see, for example, Cox, 1942; Goodale & Schwab, 1944). Although the use of penicillin has proved much more successful in this respect, moderate increases of resistance which reduce the effectiveness of penicillin treatment have recently been reported (Cradock-Watson, Shooter & Nicol, 1958).

N. meningitidis fortunately retains its almost consistent sensitivity to the sulphonamides, which have the advantage over penicillin of good penetration through the blood-brain barrier. It is to be hoped that this state of affairs will persist, for strains showing increased resistance to the sulphonamides have been reported (Schoenbach & Phair, 1948; Love & Finland, 1954).

d. Mycobacterium tuberculosis

Resistance of *Myco. tuberculosis* to streptomycin, isoniazid and *p*-aminosalicylic acid has been fully discussed and reviewed by Mitchison (1954) in this Bulletin, and by Finland

(1955b). The problem is essentially one of resistance emerging in the infective strain during treatment, and combined therapy has continued to prove highly successful in the treatment of sensitive infections by preventing or delaying the selection of resistant variants (see, for example, Ross, Horne, Grant & Crofton, 1958). Unfortunately strains isolated from new cases are sometimes resistant to one or more of the three principal agents, and occasionally to all three (Bruce, Cuthbert & Ritchie, 1957). For such infections alternative—and less satisfactory—chemotherapy by viomycin, the tetracyclines and cycloserine (Weinberger, Lindars & Pasha, 1958) is necessary; since *Myco. tuberculosis* is liable to acquire resistance to each of these drugs, they also should be used in combination.[2]

Apparent superinfection with isoniazid-resistant strains during treatment has been reported (Motiwale & Wolinsky, 1958).

3. Measures to Combat the Emergence of Resistant Pathogens

If an organism is sensitive to two or more unrelated antibiotics, and if each of them can be relied upon to eliminate the organism from an infected patient, the use of both or all of the drugs together in treatment should prevent the emergence of any but those rare mutants which achieve resistance simultaneously to both or to all of the drugs (see Bryson & Demerec, 1955). The application of this principle has been thoroughly vindicated in the treatment of tuberculosis, but there is, as yet, little information about the effect of combined therapy on the emergence of resistance in other infections. A study on the combined use of novobiocin and erythromycin in a burns Unit has shown evidence of some delay in the emergence of resistant staphylococci both in vitro and in vivo; after a few weeks of the clinical trial, however, staphylococci resistant to both antibiotics began to appear, and by the end of the third month over 20% of the staphylococci isolated in the ward were doubly resistant (Lowbury, 1957). In this study, the delay in emergence of resistant strains was associated with more effective elimination of the staphylococcus from covered burns; the break-through of resistant strains occurred in burns treated by the exposure method, which responded less well to chemotherapy. Better results might be expected in infections which are less notoriously difficult to treat; Barber, Csillag & Medway (1958), for example, found no erythromycin-resistance in staphylococci from 108 patients treated with novobiocin and erythromycin.

Other measures may help to prevent, or at least delay, the emergence of resistance in hospitals. Economy, especially in the use of streptomycin, erythromycin and novobiocin, and the restriction of prophylactic uses of such agents, are certainly effective, but not always applicable. The use of antibiotics in rotation, changing on (or preferably before) the first appearance of resistant strains, might be effective if resistance emerges after a period of delay and disappears quickly when the antibiotic in question is withheld; but it is unsafe to rely upon either of these conditions (Lowbury, 1958). Rotation of antibiotics when resistant organisms are already present in the ward does not appear to reduce the incidence of such strains (Linzenmeier & Zeit, 1959). Of much greater value is the use, whenever possible, of antibiotics against which resistance does not tend to emerge—especially in local application, for which neomycin and bacitracin are appropriate. Probably the most important measure in the campaign against resistant organisms is the attempt to prevent cross-infection.

4. Comments and Conclusions

The mounting problem of antibiotic-resistance is not reflected in the death-rate from infective disease as shown in the Registrar General's Statistical Review of England and Wales; the deaths so classified, including those from tuberculosis, show a continuous decline, with less fluctuation in recent years than formerly (see Lowbury, 1958). Some important infections, however, are separately classified, among them bronchopneumonia which does not show a similar pattern of decline during the past decade, and the Review gives no data on the incidence and mortality from hospital staphylococcal infection, against which antibiotic therapy has been least successful.

Resistant staphylococci have filled a vacuum created by the suppression of more vulnerable pathogens. Strains selected in the hospital environment include many (especially those of phage type 80) which appear to have exceptional virulence and communicability; but strains of phage group III are still the commonest hospital staphylococci, and many of them have been shown less liable to cause an invasive type of infection (Parker, 1958).

Although it is essential at present to do all that we can to preserve the usefulness of such drugs as erythromycin and novobiocin, our chief hopes must lie in the search for new antibiotics against which the staphylococci, the gram-negative bacilli and the tubercle bacillus will not acquire resistance. Recent experience with vancomycin, ristocetin, bacitracin and polymyxin, and the promising results of laboratory study with an antibiotic, cephalosporin C, that acts synergistically with penicillin against penicillinase-producing staphylococci (Florey, 1955; Crawford & Abraham, 1957), make this expectation less unrealistic than it would have seemed a few years ago.

[2] This subject is discussed more fully by Crofton on p. 55.—Ed.

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LABORATORY USES OF ANTIBIOTICS

ROBERT CRUICKSHANK M.D. F.R.C.P. D.P.H.

Professor of Bacteriology
University of Edinburgh

1 Selective culture media
2 Antibiotic-sensitivity patterns
3 Bacterial growth and variation
4 Isolation of viruses
References

In his original paper describing the discovery and properties of penicillin, Fleming (1929) demonstrated by simple techniques that this substance was selectively inhibitory to certain bacteria (mostly gram-positive organisms) and had little effect on other bacteria such as the coliform organisms. This selective action of penicillin and other antibiotics on bacteria, and their inactivity against most viruses, have been utilized in a variety of laboratory procedures; these and other laboratory uses of antibiotics will be briefly discussed in this chapter.

1. Selective Culture Media

Fleming used the selective action of penicillin on gram-positive bacteria to show that the gram-negative penicillin-resistant *Haemophilus influenzae* is present in the throats of a high proportion of healthy adults, although on ordinary culture media it is swamped by other hardier organisms like the non-haemolytic streptococci, diphtheroids, and others. He used the simple technique of dropping some crude penicillin on to one half of a plate of culture medium immediately after inoculation. This procedure was later used by two of his colleagues (i) for the easier isolation of *Haemophilus pertussis* on cough-plates of Bordet-Gengou medium (MacLean, 1937); and (ii) to isolate the acne bacillus, which tends to be overgrown by staphylococci in pustular lesions (Craddock, 1942). Later, when penicillin became standardizable, Cruickshank (1944) incorporated 0.3 unit/ml. in the Bordet-Gengou medium for the isolation of *H. pertussis* from postnasal swabs, a procedure which is now widely used.

There have been divergent findings about the sensitivity of *Mycobacterium tuberculosis* to penicillin. Some authors found that small concentrations of penicillin in a liquid culture medium seemed to enhance the growth of penicillin (Ungar & Muggleton, 1946). However, in a solid medium *Myco. tuberculosis* will withstand quite high concentrations (50–100 units/ml.) of penicillin, which has been used in plates of solid media such as Tween-albumin agar to inhibit contaminating organisms. Unfortunately, penicillin does not prevent the growth of moulds, which are troublesome on plate cultures incubated for 3–6 weeks; mycostatin or actidione may be incorporated in this or other media as antifungal agents.

The possibility that small doses of penicillin or other chemotherapeutic substances can stimulate bacterial growth has been examined by Garrod (1951) who, besides giving a critical discussion of the literature on the subject, describes some experiments of his own in which he demonstrated an accelerated growth of both sensitive (*Staphylococcus aureus*) and resistant (*Pseudomonas pyocyanea*) bacteria when subinhibitory doses of penicillin were incorporated in the culture medium.

There are examples of a more specific selective action of antibiotics in enabling the isolation and identification of bacterial species. Maxted (1953) showed that Lancefield Group A streptococci (*Streptococcus pyogenes*) was more sensitive to the inhibitory action of bacitracin than other β-haemolytic streptococci, so that this phenomenon could be used as a quick screening method for Lancefield grouping. For this purpose strips of filter-paper were soaked in a 1 in 5,000 dilution of bacitracin, dried and cut into squares which were left in a sterile container. These squares were then planted on plates of blood agar used either for primary culture or subculture of haemolytic streptococci. Maxted reported only a small proportion (1–2%) of false positive or false negative results among several thousand cultures of haemolytic streptococci, and other workers (e.g., Masters, Brumfitt, Mendez & Likar, 1958) have confirmed his findings.

Another example of a specially selective action between an antibiotic and a particular bacterial species is the resistance of *Clostridium welchii* to neomycin (Waksman, Lechevalier & Harris, 1949). This led Lowbury & Lilly (1955) to incorporate neomycin (100 μg./ml.) in Nagler plates for the suppression of most bacteria other than *Cl. welchii*, and in particular those which might give a false positive Nagler reaction. Of 3,817 meat-broth cultures from burns inoculated on to ordinary Nagler plates, 307 gave positive reactions of which 95 were proved to be false positives; on the neomycin-Nagler plates 261 were positive and none was false. This selective medium was also found useful for air sampling and for isolation of *Cl. welchii* from faeces. A slightly lower concentration of neomycin (50 μg./ml.) was found suitable for the isolation of types B, C, D, E and F *Cl. welchii* as well as for non-haemolytic variants of type A.

The selective inhibitory action of penicillin and other antibiotics may be used to demonstrate apparent antagonisms between different bacterial species. Fleming showed that when a mixture of *Staph. aureus* and a gram-negative bacillus (*Bacillus violaceus*) was inoculated in a streak on nutrient agar up to a gutter containing penicillin, the staphylococcus grew in apparently pure culture in the more distant part of the streak (where it was out of reach of the inhibitory penicillin), whereas the *B. violaceus* (being resistant to penicillin) grew in pure culture in the area nearest to the gutter. Reference is made later to the use of combinations of antibiotics such as penicillin and streptomycin for suppressing all bacterial growth, particularly in the isolation of viruses: this proceeding is also useful for the isolation of pathogenic fungi, particularly such as the slow-growing species *Histoplasma capsulatum*, from material contaminated with bacteria.

2. Antibiotic-Sensitivity Patterns

It is now common practice in clinical laboratories to submit all bacterial pathogens to a battery of antibiotic-sensitivity tests. Some of the more commonly isolated pathogens—e.g., staphylococci, coliform organisms—derived from patients in hospital are frequently resistant to one or more of the

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antibiotics in common use; the antibiotic-sensitivity pattern of these bacteria may be used as a method of identification for tracing sources and spread of infection within the hospital environment. With the five more commonly used antibiotics —penicillin, streptomycin, chloramphenicol, one of the tetracyclines and erythromycin—there is a possibility of 32 different sensitivity patterns, based on the simple assessment of "sensitive" or "resistant". If this method of "typing" is used, however, accurate quantitative assessments of sensitivity to each antibiotic are recommended. Once a particular pathogen has acquired its sensitivity pattern, its "antibiogram" remains remarkably constant, with some notable exceptions. Silberstein (1955) used this technique to trace the source of the epidemic staphylococcus in two outbreaks of post-operative wound infection; in the one incident when phage typing was also done, there was good correlation between phage and antibiogram identification. Brodie, Sommerville & Wilson (1956) used a combination of serological typing and antibiotic-sensitivity pattern in their epidemiological studies of staphylococcal infections, and Oeding & Sompolinsky (1958) have compared phage typing, serology and antibiograms in investigations of post-operative wound infections, an outbreak of staphylococcal food poisoning, and nosocomial infections in a paediatric ward. Each of the methods had its advantages and drawbacks, and the authors recommend that every laboratory concerned with the problems of hospital infection should concentrate on one method of "typing" staphylococci or other pathogens, and develop the technique to the necessary accuracy of both sensitivity and specificity. The antibiogram method is the most readily available and the findings with this technique may then be checked by phage or serological typing at a central reference laboratory.

3. Bacterial Growth and Variation

In the early studies of penicillin by the Oxford team, Gardner (1940) showed that the drug in subinhibitory concentrations induced certain bacterial species to become bloated or elongated, which suggested that growth of the bacterial cell was occurring without division and separation. Some years later Duguid (1946) studied this phenomenon and concluded that penicillin "interferes specifically with the formation of the outer supporting cell wall, while otherwise allowing growth to proceed until the organism finally bursts its defective envelope and so undergoes lysis." This remarkable deduction has been confirmed in the past few years by the brilliant work of Park (1958) and others on bacterial cell wall synthesis and its inhibition by penicillin. The tendency for bacillary forms to become greatly elongated under the influence of penicillin was utilized by Fleming and his colleagues (Fleming, Voureka, Kramer & Hughes, 1950) in studies on flagella and motility, when with an ingenious technique it was shown that flagellar activity was responsible for the propulsion of the elongated and curled-up forms of *Proteus* and that this activity was very sensitive to heat stimulation.

Antibiotics have also been used for the isolation of bacterial variants such as the small colony G forms of the staphylococcus. The culture is grown in a concentration of penicillin which is just sufficient to inhibit growth of the normal forms, and the small G colonies can be studied by maintaining cultures at this level of antibiotic. A more dramatic effect of penicillin in converting *Staph. aureus* to a gram-negative bacillary form was first reported by Klimek, Cavallito & Bailey (1948) and has been further studied by Crawford & Abraham (1957) and by Briggs, Crawford, Abraham & Gladstone (1957). The gram-negative bacillary variant had biological characters and antibiotic sensitivities in keeping with other gram-negative bacilli, and most of its characters were completely different from the parent staphylococcus. Yet staphylococci identical with the parent strain could often be recovered from the gram-negative variant when subcultured in the absence of penicillin. These and similar studies with other antibiotics (see Voureka, 1951, 1952) may have some significance in the isolation and identification of bacterial pathogens from the lesions of patients under antibiotic therapy. They also shed light on some of the problems of bacterial metabolism and mutation.

Another laboratory procedure, called the "penicillin technique", has been used by bacterial geneticists for some time for the isolation of auxotrophic members of mixed bacterial populations. It takes advantage of the fact that penicillin kills only growing cells, so that when a mixture of "wild" and auxotrophic cells is grown on a minimal medium containing penicillin, only the wild variants grow and are killed, and the auxotrophic variants can then be grown by adding a growth factor such as an amino acid (Lederberg & Zinder, 1948; Adelberg & Myers, 1953).

4. Isolation of Viruses

It is perhaps fortunate that the great technical advances in the cultivation of viruses have coincided with the development of antibiotics that are inhibitory to most bacterial species but not to viruses (with a few exceptions), and at the same time are non-toxic to living cells. In the pre-antibiotic era, isolation of viruses from material that contained bacteria, e.g., throat washings from suspected cases of influenza, could only be achieved after passage through fine filters or by high-speed centrifugation. Today, specimens of faeces, throat swabs and other bacteria-contaminated material are "treated" with a mixture of penicillin (100 units/ml.) plus streptomycin (100 μg./ml.) before being inoculated into various types of tissue culture, or into chick-embryos or suckling mice. If it is suspected that the material may contain penicillin-resistant or streptomycin-resistant bacteria, neomycin in a strength of 50 μg./ml. is used; higher concentrations may be toxic to the tissue cells. Neomycin may also be used to inhibit the development of pleuropneumonia-like organisms that sometimes contaminate tissue cultures. Mycostatin (100 units/ml.) may be used to prevent growth of yeasts or fungal contaminations which periodically cause trouble in tissue cultures.

A few of the larger viruses, e.g., the psittacosis–lymphogranuloma group, are sensitive to penicillin and the broad-spectrum antibiotics, so that the addition of these drugs to material suspected of containing members of this group may prevent their growth. Thus, the early failures to cultivate the virus of trachoma may have been associated with the antiviral action of penicillin. Because of this, Collier & Sowa (1958) used streptomycin alone (1,000 μg./ml.) as a bacterial inhibitor in their successful attempts to cultivate this elusive virus.

Small concentrations of penicillin and streptomycin are also added to tissue cultures in the large-scale production of virus vaccines, e.g., poliovirus vaccines, but only minimal

traces of these antibiotics appear in the final product. Nonetheless, rare instances of hypersensitivity reactions in penicillin-sensitized individuals have been reported following injections of poliomyelitis vaccine. The addition of antibiotics to calf and sheep lymph in the harvesting of smallpox vaccine has also been recommended, but glycerol is apparently more generally effective in getting rid of the usual bacterial contaminants in such material.

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THE SEARCH FOR NEW ANTIBIOTICS

A. H. CAMPBELL B.Sc. Ph.D.

Glaxo Laboratories Ltd.
Stoke Poges, Buckinghamshire

1 Primary screening in vitro
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During the past fifteen years the discovery of several thousand new antibiotics has been recorded. Those which have shown sufficient promise to justify the work of characterization number about three hundred, but only a dozen have passed into general use. Today, there is still a need for new antibiotics both to expand the range of therapy and to combat the emergence of resistance to established antibiotics on the part of some bacterial pathogens.

The earliest targets set were the bacterial pathogens unaffected by penicillin, screens being set up to search particularly for broad-spectrum and antituberculosis substances. Soon they were augmented to select antibiotic activity against fungi and protozoa, while more recently interest has centred on substances active against viruses and tumours. Problems of staphylococcal resistance to the range of commonly used antibiotics have given renewed impetus to the search for new substances effective against gram-positive cocci. Outside the fields of human and veterinary medicine, attempts are now being made to select antibiotics for use against pests and pathogens of plants, such as mites, insects, pathogenic fungi and nematodes.

1. Primary Screening in Vitro

The basic technique for screening antibacterial substances was derived from Sir Alexander Fleming's classical agar plate by replacing the chance contamination by the *Penicillium* colony with previously produced metabolic products of a variety of unknown organisms, usually isolated from the soil. To a large agar plate seeded with test bacteria, the metabolic products are introduced as culture broth dropped into holes in the agar or as plugs cut from cultures on solid medium. This kind of work, a simple demonstration of antibiosis, is readily accomplished, and many microbiologists all over the world have engaged in this fascinating exploration of antibacterial action. However, it must be realized that an in-vitro demonstration of antibiosis is commonplace. Many obstacles, such as inactivation in the body and unacceptable toxicity, block the way ahead of the newly discovered antibiotic. The remarkably low mammalian toxicity of penicillin was the fortuitous element which distinguished the discovery of penicillin from earlier demonstrations of antibiosis. In all too many instances, the development of an antibiotic with important new properties has been barred in the end by a toxic hazard only discernible after prolonged and intensive toxicological studies.

Even nowadays, when we have grown accustomed to the research team, the search for new antibiotics is outstanding in the number and variety of scientific disciplines involved. Microbiologists, chemists, pharmacologists, physiologists, pharmacists, engineers and technologists all must contribute before a new antibiotic reaches the clinician for trial. Thereafter, should manufacture be contemplated, intensive development must be undertaken to increase, by mutation and strain selection, the originally meagre yield of antibiotic. Improvements of the culture medium and of the extraction and purification processes must proceed simultaneously. Indeed, there could hardly be a greater contrast between the simplicity of the discovery of antibacterial action in vitro and the complexity of the investigations into efficacy and safety required before the new substance is offered for clinical use.

2. Basic Screening Methods

As must be expected, screening methods vary considerably in complexity from one laboratory to another, but the basic principles can be illustrated by considering as an example the screening of streptomyces species against staphylococcus. The choice of *Staphylococcus aureus* as test organism is based on considerations such as its importance as a systemic pathogen and the fact that many strains now show resistance to some extent to currently available antibiotics. Artificial infections of laboratory animals can be reliably produced with staphylococcus to provide in-vivo protection tests for evaluating new antibiotics thrown up by the screen. The choice of streptomyces as antibiotic producers derives from the large numbers of antibiotics already known from this group, their availability in large numbers in the soil, and the wide variety of the morphological and biochemical characteristics exhibited.

a. Primary Screening

This is the simplest step, consisting of the isolation of the unknown streptomyces and the selection of those which exhibit antibiotic action for further study. For this, samples of soils, frequently obtained by a world-wide collection system, are shaken up with sterile water and plated out through a series of dilutions on two or more different media dispensed in Petri dishes. After incubating for seven days, the colonies of streptomyces are ready for "picking off" from the mixed growth comprising moulds, yeasts, streptomyces and bacteria (Plate III, A). This is done by transferring each selected colony to a nutrient agar slope which then becomes the reference culture for all subsequent work.

To examine for antibiotic activity, subcultures are then made in small aluminium cups containing a variety of nutrient agar media. After four days' incubation, plugs are cut out of this culture with a cork borer and placed on large plates of agar medium seeded with the test organism—in this case, *Staph. aureus*. Following incubation overnight at blood temperature, the plates are examined for the characteristic clear zones of inhibition around the culture plugs, indicative of antibiotic activity (Plate III, B). In this first demonstration of antibiosis, it is customary to include test plates seeded with a variety of bacteria to gain further information on antibacterial activity other than against the target staphylococcus.

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(FIGS. A, B)

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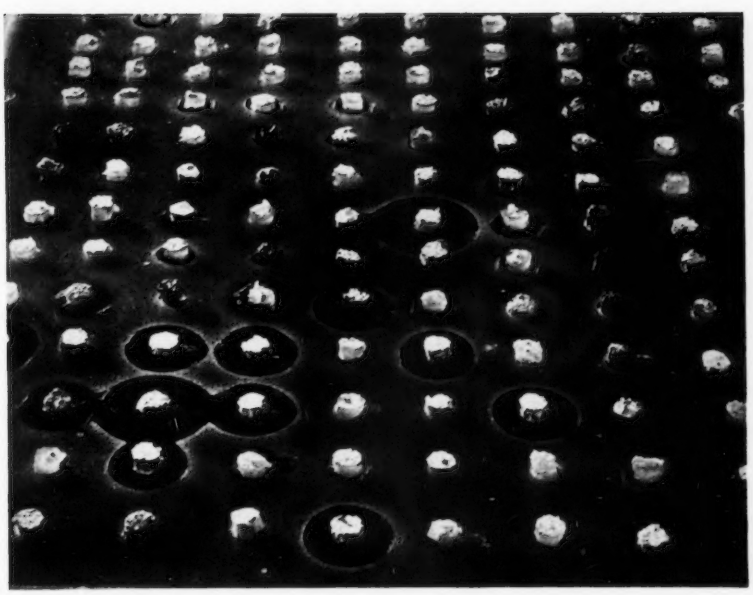
A. Plates poured from soil dilutions, showing mixed cultures of moulds, yeasts, streptomyces and bacteria, with the isolated strains in the reference cultures
(magnification × 0.25)



B. Demonstration of antibiosis. Plugs taken from agar-plate cultures of the isolates are incubated on the surface of a nutrient plate seeded with the test organism. The clear dark zones result from antibiotic activity in the plugs
(magnification × 0.25)

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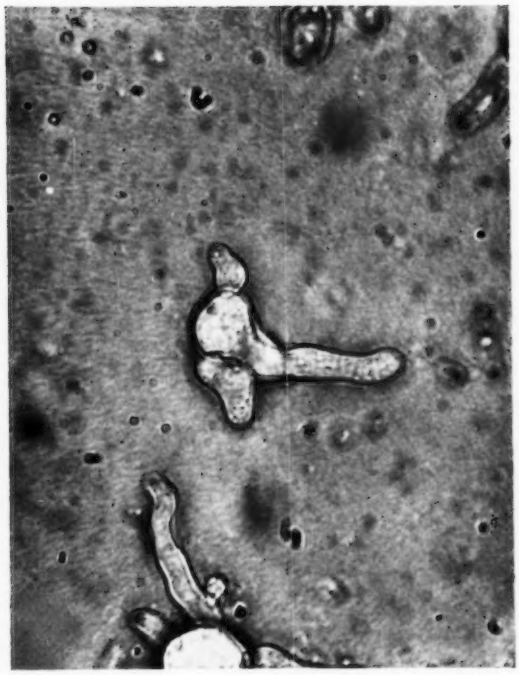
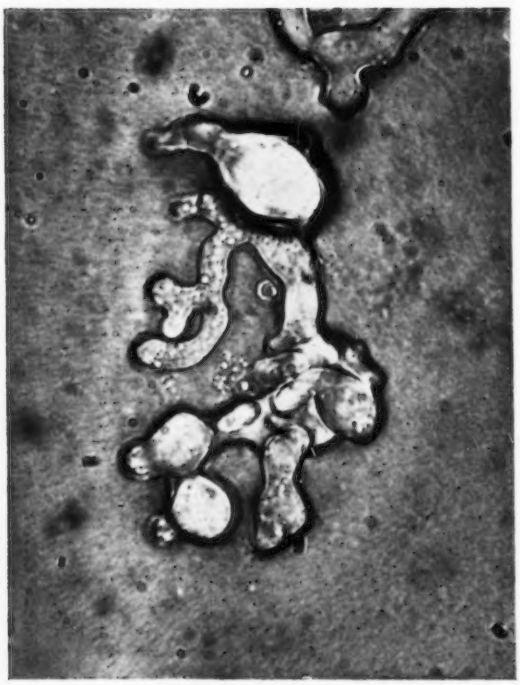
C. Spores of *Microsporum canis* germinating on Sabouraud's agar containing no griseofulvin; 18–20 hours' growth at 25° C.

(magnification × 200)



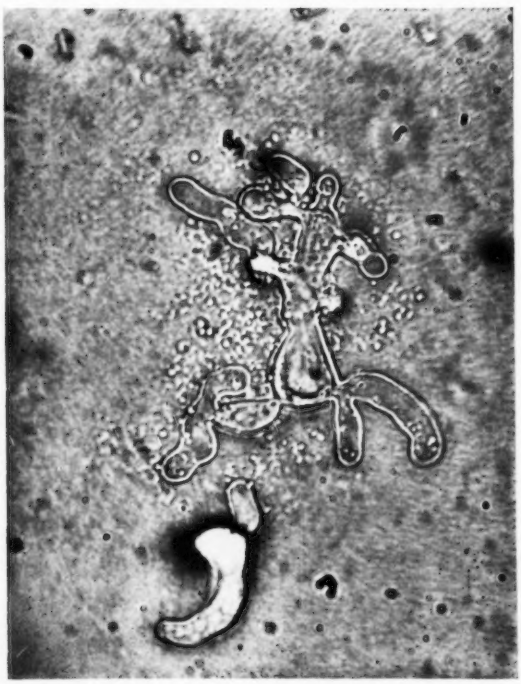
D. Spore of *Microsporum canis* germinating on Sabouraud's agar containing 10 μg./ml. griseofulvin. This represents the total growth achieved in 84 hours' incubation. Compare size of original spore and hyphal width with fig. C

(magnification × 200)



E. Colony derived from a spore of *Microsporum canis* germinated on Sabouraud's agar containing 10 μg./ml. griseofulvin. Hyphae are grossly distorted and have large bulbous vesicles and swellings. A small swelling in the centre of the colony has burst. 120 hours' growth at 25° C.

(magnification × 200)



F. The ultimate fate of a colony of *Microsporum canis*. A colony after 156 hours' growth on Sabouraud's agar containing 10 μg./ml. griseofulvin at 25° C., showing the empty burst hyphae with the extruded protoplast scattered around

(magnification × 200)

b. Secondary Screening

With the demonstration of antibiosis for a particular isolate, the reference culture is passed to the secondary screen. Here the first objective is to establish the conditions for submerged culture in flasks aerated by shaking, to ensure adequate quantity of the antibiotic. This achieved, a further study of the antibiotic is made on large assay plates to select the most potent amongst those available. A test is also run on medium containing 10% whole blood to eliminate immediately those antibiotics inactivated by blood.

Thereafter, the selected isolate is fermented in larger bulk to provide the extraction chemists with sufficient material to prepare aqueous concentrates suitable for animal testing. Usually, the extractions used are of a simple physical type such as solvent extraction followed by regeneration in water, charcoal adsorption and elution, use of ion-exchange resins and precipitation by pH change or ammonium sulphate addition.

The aqueous concentrates so prepared are now submitted to the first vital hurdle by being injected into mice, intraperitoneally for toxicity measurement and subcutaneously for testing protection against virulent staphylococcal infection. The resulting information provides the first index of likely value, namely, the toxicity-protection dose ratio (i.e., the ratio between the lethal dose and that required for therapeutic effect); this is usually arbitrarily set at 10:1—a value which eliminates 9 out of 10 of the isolates entering the secondary screen.

Assuming now that an antibiotic has successfully passed through the secondary screen with a favourable toxicity-protection dose ratio, the next step is to attempt a rapid identification based on the crude product. To achieve this with certainty is most difficult, yet it is obviously desirable since the extraction and purification of antibiotics requires much expenditure of effort which should be reserved for new substances. Some help may be gained from what is known about the chemical properties or the characteristics of the organism producing an antibiotic, but most reliance must be placed on two techniques which have been highly developed for this purpose.

The first is the comparison with known antibiotics by paper-strip chromatography. The modification employed in antibiotic work involves the bio-autograph, whereby the extent and position of the antibiotics developed on the paper strips are demonstrated by placing the strips on the surface of a large seeded agar plate and incubating until the antibiotic activity against the test organism is manifested by the appearance of clear zones.

The second method uses the demonstration of cross-resistance with known antibiotics. For this purpose, resistance to known antibiotics is built up in the test organism by cultivation in the presence of increasing concentrations until it is at least 100 times greater than that of the normally susceptible test strain. A comparison is then made of the activity of the unknown antibiotic against the susceptible strain and against strains made highly resistant to known antibiotics.

Unfortunately, the complexity of the interrelationship between antibiotic-resistant organisms is such that a finding that a particular unknown is cross-resistant with a known antibiotic is not by itself diagnostic. This is because, while there are families of antibiotics which are reciprocally cross-resistant, there are many others with one-way cross-resistances. However, by extending the observations into a variety of test species it is possible to record a spectrum of cross-resistance which, when used in conjunction with the data from the paper chromatogram, permits the classification of the new antibiotic as one of the known antibiotics, closely related to one of them, or as an unknown entity. With this information and having regard to all other characteristics disclosed by the screens, it can then be decided whether or not to undertake the isolation of the antibiotic in the purest form possible. This purified product then becomes the basis of the detailed toxicological and pharmacological investigation preceding any clinical evaluation.

Probably the greatest difficulty arises when the newly discovered antibiotic is held to be not identical with but related to one already known. It is not generally realized that, amongst the metabolic products of micro-organisms, the same basic structure is frequently encountered with small differences in chemical constitution. Most of the well-known antibiotics are single members of families of closely related substances. Within each family there is a similar, if not identical, range of biological activity. Frequently, the different members of the family are produced by morphologically distinct strains, then commonly elevated to the status of new species, but in many other instances a single strain produces a whole family of related antibiotics under varying conditions of cultivation. An early example is given by penicillin, where, at one time, the recognition of a half-dozen different penicillins in the culture broth posed serious problems in purification. These, however, were swept away by the discovery that incorporating the benzyl precursor, phenylacetic acid, in the medium resulted in the production of penicillin G almost exclusively. Amongst other well-known families are the tetracyclines and the more extensive macrolides comprising erythromycin, oleandomycin, and a number of others.

The last five years, in which there has been a marked slowing down in the rate of discovery of important new antistaphylococcal antibiotics, have seen an intensive re-investigation of the families of known antibiotics. Although in most cases this has resulted more in an access of knowledge of antibiotic structure than in clear clinical advantage in the new-comers, such work will undoubtedly be continued in the hope of discovering new products with distinctive properties.

Another facet of the search for new antibiotics among existing families is particularly interesting because of the combination of chemical synthesis with biological production of a related compound with the basic structure of the molecule. The recently publicized isolation of the "penicillin nucleus", or 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959), illustrates an approach which may well be followed with other families. Although the existence of the penicillin nucleus was held to be demonstrated many years ago, these workers have isolated the compound in sufficient quantity to prepare some 200 new penicillins, but without—at the date of writing this paper—finding one with outstanding merit. Meanwhile, following the total chemical synthesis of penicillin V, reports have appeared of the synthesis of new penicillins, one of which, although it had activity against penicillin-resistant staphylococci, was not judged worthy of commercial development (*New York Times*, 1959).

3. Primary Screening in Vivo

The position in medicine won by the antibiotics in the treatment of bacterial diseases has not so far been attained by

comparable research into the use of antibiotics against virus diseases and tumours. The antibacterial screen has had an exceedingly potent tool in the simple in-vitro demonstration of antibiotic activity which allows the economical screening of many thousands of new isolates each year, facilitating further study and isolation of the new antibiotics produced.

When virus diseases were put up as targets for antibiotic screening, it was to be expected that reliance should first be placed on the familiar methods so productive of antibacterial substances. Only in this way could the vast number of new isolates deemed necessary be effectively handled. Working on these lines, the most convenient test was to examine the effects of crude preparations of new antibiotics in protecting susceptible bacterial cells from the action of bacteriophage. Little success attending these efforts, the method was soon abandoned as having too little relevance to the treatment of virus diseases in man. However, the convenient mechanics of an in-vitro screen seemed to many to be of paramount importance, and other methods were explored in detail.

In particular, the technique of tissue culture was advocated by some as an effective replacement for the more cumbersome procedures using developing chick-embryos and mice. Perhaps the best known is the technique where films of growing human epithelial HeLa cells, susceptible to poliomyelitis virus, show characteristic plaque formation from single virus particles.[1] Tissue films are infected by the virus and then covered with a thin layer of agar, and filter-paper disks impregnated with the metabolic products of the isolates being screened are placed on the surface of the agar. If a disk is surrounded by a zone of healthy cells, not damaged by the virus, while elsewhere there are numerous spots of necrosis, then an antiviral action has been demonstrated (Dulbecco & Vogt, 1954).

Certainly, a large number of antibiotics as well as synthetic organic compounds have been reported to have antiviral activity against one or more viruses growing in tissue culture or embryonated egg, but the correlation against the same virus in the intact host has been very poor. To many, recent work on viruses makes it appear that they are too different from bacteria for comparable methods to be used and that, in particular, the susceptibility of a virus growing inside the cell may be quite different from that of the virus outside the cell. To extrapolate from a positive finding in a contact in-vitro test to the likely value of the substance as a chemotherapeutic agent is fraught with known as well as very likely unknown difficulties.

There remains among investigators a marked difference of view as to a preferred method of antiviral testing; the tissue-culture and embryonated-egg test being capable of high throughput, while the clearer relevance of the infected mammal test is offset by its laborious nature and high cost.

The search for a chemotherapeutic action in viral infections now seems closer to the search for antitumour agents than that for antibacterial substances. As such, to provide relevant in-vivo testing matched in numbers to the familiar antibacterial screen, it will need the service of as large resources as are currently being given to cancer research. However, since a number of animal neoplasms are known to be induced by virus, it is not outside the bounds of possibility that an effective antiviral agent may be derived from the present mammoth cancer campaign.

[1] This and other tissue-culture techniques used in the study of viruses are described by J. C. N. Westwood, *Brit. med. Bull.* (1959) 15, 181.—ED.

The search for antitumour agents originally began with the testing of the products of the organic chemical laboratory and has been extended to include antibiotic substances. The reasoning has been that the advent of antibiotics disclosed a variety of chemical structures previously unsuspected and that the extreme subtlety of their effect is manifest in the little understanding we have today of their precise mode of action. As is appropriate to the magnitude of the problem and the meagre practical benefits which have so far come out of many years of cancer research, the approach to the testing of antitumour agents is being conducted on the widest possible front. At least as much effort is now being expended on research into methods of testing as putting new substances through an existing screen. The agents which have so far found limited use have shown a degree of general toxicity which contrasts strongly with the remarkable degree of specificity characteristic of the antibiotics. It may well be through this selectivity that the best hope for the discovery of an effective antitumour agent lies.

In the search for new antibiotics the empirical method of screening unknown substances has been extremely successful in the past, as witness the major extensions in antibiotic therapy achieved. As well, there are indications that a rational approach to new antibiotics will be more extensively pursued in the future, probably along the lines of partial synthesis such as with the penicillin nucleus or by total synthesis of polypeptides, as has already been shown for gramicidin S and some of its analogues (Schwyzer & Sieber, 1956). That there are other possibilities is evident from the recent development of griseofulvin from a laboratory curiosity to the first systemic agent effective against dermatophytes.

The story of griseofulvin from the time of its first isolation as a crystalline substance twenty years ago (Oxford, Raistrick & Simonet, 1939) is now familiar to many (see *Lancet*, 1958). Its importance in the present context is that griseofulvin's status owes nothing to the screening of unknown substances. In fact the fungistatic action is of such an extraordinary kind that the selection of griseofulvin as an antifungal antibiotic would have been most unlikely from its demonstrable activity on an assay plate. Instead, the conventional screens uncovered numerous members of the polyene group of antifungal substances, none of which have proved of value against the common fungal diseases caused by the dermatophytes.

The dermatophytes live and multiply in what is virtually dead tissue, their persistence in the skin depending upon keeping pace with the rate at which the superficial keratin is shed. Griseofulvin, taken by mouth, appears in the keratinized cells, endowing them with the power to prevent penetration by fungal hyphae (Gentles, Barnes & Fantes, 1959). The way in which this happens can be deduced from the hyphal changes brought about by very low concentrations of the antibiotic. When as little as 0.1 μg./ml. is added to the growth medium, the hyphae enlarge and curl, showing a disorientation which, with higher concentrations, leads to gross deformity and lysis (Plate IV, D–F). This effect, exerted at the tips of the growing hyphae, prevents the penetration of the newly formed griseofulvin-containing keratin, leading to the fungus being ultimately shed with the infected layers of skin.

For the development of griseofulvin, the clues lay in the nature of the disease process, the exceedingly low mammalian toxicity together with the affinity of the antibiotic for keratinous cells, and the fungistatic action evident on the growing

tips of the hyphae. Thus, to extend the present range of antibiotic therapy, it may well not be enough to rely on the mechanics of a screen. There is a need for a biological curiosity about the nature of the antibiotic action disclosed, as well as a full understanding of the whole disease process. Might it not be that this could restore to the individual worker the power to make an antibiotic discovery fit to rank with Fleming's penicillin?

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Book Reviews

Heterocyclic Chemistry: An Introduction

Adrien Albert. London: Athlone Press, 1959. viii + 424 pages; 26 figures. 22 × 14 cm. £2 5s.

The author, who is Professor of Medical Chemistry at the Australian National University, has achieved a remarkable feat: he conveys, in one volume and without excessive compression, a good general idea of heterocyclic chemistry. This has been done by classifying heterocyclic systems according to their electronic character, and by discussing their properties largely in that light. The high quality of the book reflects the soundness of the approach. The selection of details to exemplify the theme has naturally been influenced by personal experience. Three special chapters are devoted to physical properties, which are also well to the fore in other parts of the book. Ultra-violet absorption spectra receive thorough treatment, somewhat at the expense of the infra-red range, and useful tables of ionization constants, dipole moments and oxidation-reduction potentials have been compiled.

The problems of tautomerism (keto-enol and lactam-lactim), so often encountered in heterocyclic chemistry, are fully discussed, and the author has much of value to say on the methods available to solve them. Occasionally, however, it has been overlooked that certain facts—e.g., the existence of *cis-trans* isomerism in succinic anhydrides and succinimides, and of optical activity in oxazolones—have already decided questions of tautomerism not investigated by newer methods.

In my opinion, insufficient attention has been paid to small-ring heterocycles; to polysaccharides or glycosides, which are not mentioned even as examples of biologically important heterocyclic compounds; and to the detailed mechanism of reactions. However, so much has to be left out in a book of this length that a critic's list of omissions is as likely to reflect personal choice as the author's list of topics to be included.

Although the emphasis throughout is on properties rather than on preparation, sufficient indication is given of the principal syntheses of all heterocyclic systems. The author keeps in mind any industrial, biological or theoretical interest of the substances discussed. For the reader wishing to have more details of a particular subject, frequent references are made to comprehensive reviews.

The book is excellently produced, with clear pictorial formulae, and is agreeably free from typographical errors. It can be recommended to advanced students of organic chemistry, and to workers in biology or medicine who wish to understand the chemistry underlying some of the most important constituents of living matter.

J. W. Cornforth

Staphylococcus Pyogenes and its Relation to Disease

Stephen D. Elek. Edinburgh: E. & S. Livingstone Ltd., 1959. vii + 767 pages; 44 figures. 25 × 16 cm. £4 4s.

This book can only be described as a tour de force. It embodies the information gleaned from approximately 4,000 sources, ranging from the pioneer work of Sir Alexander Ogston in 1880 to papers published as recently as 1957. In the preface the author apologizes for failing to provide a comprehensive bibliography, but few readers will complain that the references cited are insufficient. For many this vast literature dealing with staphylococci would leave little time for other reading, but the present author's catholic literary taste is reflected in the provocative and diverting quotations which head the chapters.

Practically everything known about the staphylococcus appears to have been mentioned, and I defy anyone, however conversant with this microbe, to read the book without learning something unexpected. How many bacteriologists know, for example, that the growth of staphylococci is inhibited by the royal jelly of the honeybee and by a substance obtained from the green alga *Chlorella vulgaris*, or, that staphylococcal extracts reduce the number of takes in experimentally transplanted mouse carcinomata?

After a brief but entertaining historical survey the author plunges into the problems of taxonomy in relation to a microbe of which it is rightly said that "Almost all its typical characteristics are subject to frequent variations". Then follow chapters on morphology and staining reactions, metabolism, antigenic structure and serological classification, staphylophages and lytic phenomena, distribution of *Staphylococcus pyogenes* in nature and staphylocoagulase. All are packed with information and draw attention to interesting and much-neglected studies of earlier decades as well as those of modern times. Particular mention should be made of the chapter on metabolism, which is an excellent exposition of a difficult subject.

Chapter IX, devoted to diffusible products of staphylococci, is of special interest since the author's own work in this field is of some importance. This and the two following chapters, which discuss the problems of virulence and resistance in relation to staphylococcal disease, offer much food for thought, although some of the author's views might be questioned by other workers. Thus on p. 338 he concludes, from a series of experiments which ignore variations in the invasive capacity of staphylococci, that ". . . gross differences in the virulence of coagulase-positive strains are not likely and that other factors determine the occurrence of clinical disease". Nevertheless these three chapters represent an important contribution to the subjects discussed and deserve careful study.

The next three chapters, concerned with antistaphylococcal agents, make rather heavy reading. Of course, such subjects as the mode of action of antibiotics are not easily presented in a simple form, but over-condensation and immensely long paragraphs (often more than a whole page in length) do not help. In parts there is definite confusion. For example, in the discussion of penicillin-resistant staphylococci it is not always clear whether penicillinase-producing or drug-tolerant strains are being referred to.

The book concludes with a chapter on clinical problems to which the author has an unusual and interesting approach. He stresses the fact that "The more successful parasites are the ones which can often establish symbiotic existence, or the carrier state, without causing recognisable disease", and suggests that our main preoccupation should be to discover what goes wrong when these "hangers on" cause disease.

No short review can do justice to this book, which, whatever else is said about it, is a monumental piece of work. From the outset it provokes contradiction. Thus how many hospital bacteriologists would support the view that in this era of antibiotics ". . . staphylococci . . . have lost much of their terror"? The book is sometimes confusing, and occasionally contradictory statements are made in different chapters (cf. statements on mouse

susceptibility to intravenous and intraperitoneal infection, pp. 336, 361, and statements on variations in virulence, pp. 338, 507). Nevertheless anyone interested in the staphylococcus will find the book a mine of information and, although sometimes provoking, always stimulating.

Mary Barber

Biochemistry of Antibiotics: Proceedings of the Fourth International Congress of Biochemistry, Vienna, 1–6 September, 1958. Volume V, Symposium V

Edited by *K. H. Spitzy & R. Brunner*. London: Pergamon Press, 1959. xii + 239 pages; plates; figures. 24 × 16 cm. £3 8s.

One of the Symposia held during the Fourth International Congress of Biochemistry in Vienna during the summer of 1958 was on the subject of the biochemistry of antibiotics. This volume contains the 19 main contributions given at the Symposium, together with some of the comments made during discussion of these contributions. The subject is an immense one and, although comprehensive treatment is not possible on such an occasion, the organizers of the Symposium have collected together a series of contributions representing the main branches of the subject. Topics include the classification of antibiotic-producing *Actinomycetes* and accounts of the chemistry, biogenesis, properties, methods of estimation, toxicology, pharmacology, interactions, and structural interrelationships of various antibiotics. The biological interactions leading to cross-resistance, synergism and antagonisms are discussed in a number of papers, as are the biochemical bases for resistance and modes of action. The possibility of extending the "antibiotic approach" to the problem of cancer therapy is examined and the medical issues raised by the use of antibiotics for food preservation surveyed. The volume concludes with a summarizing report on the Symposium by E. B. Chain, 10 of the 20 pages of this report being taken up by chemical formulae of a wide range of antibiotics. The volume as a whole provides a useful guide to many of the advances and problems in a complex field, and each of the main contributions concludes with a list of references to the original literature. There is unfortunately no index.

Ernest F. Gale

Modern Drug Treatment in Tuberculosis

J. D. Ross. 2nd ed. London: Chest and Heart Association, 1959. 50 pages. 20 × 14 cm. 5s.

This small book is intended primarily for the instruction of nurses; and for this purpose it is an admirable, concise and clearly expressed account of chemotherapy as practised and taught in many centres in Great Britain. It deals briefly but adequately with the fundamental principles that govern treatment—mode of action, drug resistance and toxicity. Regimens of treatment, combinations of drugs and dosages are described dogmatically, as is desirable in such a handbook; and the less important drugs —such as viomycin, pyrazinamide, cycloserine—receive sufficient attention. The desire to be up to date has perhaps outstripped prudence at one point, where it is stated that 0.25 g. twice daily of α-ethylthio*iso*nicotinamide appears a clinically effective dose; for the published evidence hardly justifies such a claim.

For doctors who deal only occasionally with tuberculosis and for medical students the book should also be useful. If treatment were always carried out along the lines prescribed by Dr Ross more lives would be saved, there would be fewer failures of chemotherapy, and consequently less danger to the public health. Those who wish to have a fuller exposition, with indications of the evidence on which the dogmatic statements are based, could refer to Dr Ross's article in *Tubercle*, 1958, **39**, 45.

J. R. Bignall

Modern Trends in Pathology

Edited by *Douglas H. Collins*. London: Butterworth & Co. (Publishers) Ltd., 1959. x + 334 pages + 12 index pages; 135 figures. 25 × 17 cm. £3 10s.

The pattern of this book has become familiar from productions by these publishers in other fields of medical activity. Professor Collins has collected an interesting team whose contributions range over a wide field. Sir Roy Cameron contributes a characteristic account of the electron-microscopic studies of impaired cell function. R. J. V. Pulvertaft summarizes his long experience in the examination of pathological material by cultural methods. D. M. Angevine discusses injury to connective tissue at the experimental level. C. L. Oakley surveys recent work on the site of antibody production, and W. G. Spector tackles the intricate problem of what he refers to as the "autopharmacology" of inflammation. Still on the experimental side are accounts by R. H. Mole of the effects of whole body irradiation; and by G. Payling Wright of the movements of toxins and viruses in the nervous system.

In the field of tumour pathology are chapters by R. A. Willis on some unusual tumours, J. N. P. Davies on cancer in Africa, P. E. Steiner on cancer of the liver, and A. C. P. Campbell on carcinoid tumours and 5-hydroxytryptamine. Finally there are chapters on the pathology of the human trophoblast by W. W. Park, the adrenal cortex by T. Symington, occupational lung disease by J. Gough and pathological ossification by the editor in collaboration with R. C. Curran.

It is inevitable in a book of this type that the individual reader will find certain chapters of absorbing interest and the rest perhaps of only passing concern. To the present reviewer those sections on whole body irradiation, cancer in Africa, 5-hydroxytryptamine, occupational lung disease and pathological ossification proved particularly fascinating; but this is merely an indication of personal interests, and all the chapters are of undoubted value to the teacher and worker in a broad field.

It is equally inevitable that a book of this type will have a rather short life before it becomes too out of date to merit its title. One questions the wisdom of the publishers in applying such a high standard of material and production to the volume, for this necessitates its being sold at well over twice the price of a current copy of the *Journal of Pathology and Bacteriology*, though the two contain about the same amount of material. Would not a lower-priced "paper-back" on less expensive paper meet the need for these current reviews better? This, however, is an economic, rather than a scientific, criticism.

T. Crawford

World Medical Research: Principles and Practices

Harry Sutherland Gear. London: Butterworth & Co. (Publishers) Ltd., 1959. xii + 117 + 5 index pages. 26 × 19 cm. £1 10s.

Nowadays a great deal of lip-service is paid to research. Modern methods of dissemination of news have given many people the idea that science has much to offer them in their everyday lives. This is, in itself, no bad thing. But its corollary is that with growing public interest there is growing political interest in research; and when this inevitable change is coupled with the growing costs of supporting research, costs which can be met only out of the public purse, there is a real need to ensure that the research administration is sensibly based.

Dr Gear tells the story of the role of the World Health Organization (WHO) in medical research against a background of his own thoughts on the history of man's endeavour. He has been associated with policy-making in WHO for a number of years and is consequently an experienced administrator of medical research; and his views are thus of great interest. His book is a strange mixture of liberal ideas and of administrative clichés. When he discusses the motives of WHO he does so in the language of the political document. The objectives of international co-operation are to create a "favourable climate" for research; to advance scientific and medical knowledge. These are, of course, noble sentiments but vague instructions. Research flowers in the most unlikely soil, and advances flow from the strangest springs.

At many points in his narrative one is brought sharply to realize that Dr Gear is essentially a liberal man and a knowledgeable man. He is aware that research is essentially the purview of the individualist and quotes from G. M. Trevelyan a text for all scientific —indeed for all—administrators: ". . . the very highest products of the human mind are the outcome of choice and freedom and variety rather than of uniform organization".

It must be hoped, that those responsible, as laymen, for the distribution of money to support research, will always bear this in mind. It is a text that indeed should hang over all their desks. The difficulty of working as a cog in a large machine without losing sight of this essential attitude is perhaps one reason why, in his fascinating book, Dr Gear seems somehow not wholly clear as to what precisely he wishes to say.

W. L. M. Perry

Notes on Contributors

DR E. P. ABRAHAM has been a Fellow of Lincoln College, Oxford, and a senior research officer at the Sir William Dunn School of Pathology, Oxford, since 1948. He was a Rockefeller Foundation Travelling Fellow at the University of Stockholm in 1939 and at the University of California in 1948. He gave the Ciba Lectures in Microbial Biochemistry at Rutgers University, New Jersey, in 1957. He collaborated with Professor E. B. Chain, Sir Robert Robinson and others in the work done at Oxford on the chemistry of penicillin. His subsequent work, published chiefly in the *Biochemical Journal*, is concerned with the chemistry and biochemistry of peptide antibiotics and other naturally occurring peptides with biological activity. He has contributed to *Antibiotics* (H. W. Florey *et al.* 1949) and to *General pathology*, edited by H. W. Florey (2nd ed., 1958). He is a Fellow of the Royal Society.

PROFESSOR G. A. H. BUTTLE was a pharmacologist at the Wellcome Physiological Research Laboratories for fifteen years and Advisor in Blood Transfusion in the Army in the Middle East and north-west Europe from 1939 to 1946. He is at present Wellcome Professor of Pharmacology at the School of Pharmacy, London. His interests in chemotherapy were the treatment of streptococcal infections of mice with *p*-aminobenzene sulphonamide (*Lancet*, 1936, **1**, 1286, with W. H. Gray & D. Stephenson), the action of sulphanilamide in clinical infections, and the mechanism of its effect (*Lancet*, 1936, **2**, 1323, with L. Colebrook & R. A. Q. O'Meara). He has also been interested in antibiotics and in general pharmacology. Professor Buttle is now interested in the growth of human tumours transplanted into rats and hamsters.

PROFESSOR E. G. L. BYWATERS is Honorary Director of the Medical Research Council's Rheumatism Research Unit and Honorary Consultant Physician at the Canadian Red Cross Memorial Hospital, Taplow, Buckinghamshire, and holds the Chair of Rheumatology at the Postgraduate Medical School of London. During the Second World War he was Deputy Director of the Medical Research Council's unit for research in shock, at Newcastle-upon-Tyne, and a member of the Medical Research Council's team for investigation of air-raid casualties in London. As a Rockefeller Travelling Fellow, he worked at the Massachusetts General Hospital with Dr Walter Bauer on problems of the physiology of joints; and as a McKenzie-McKinnon Research Scholar he worked under Professor E. C. Dodds at the Courtauld Institute of Biochemistry on the metabolism of tissues of joints. At present he is engaged on studies of the natural history, pathology and aetiological aspects of rheumatic diseases in children and adults. Among many published papers are the following: "The metabolism of cartilage" (*Nature, Lond.* 1936, **138**, 30); "Crushing injury" (*Brit. med. J.* 1942, **2**, 643); and a paper on the relation between heart and joint disease (*Brit. Heart J.* 1950, **12**, 101). He has previously contributed to the Bulletin papers entitled "Ischaemic muscle necrosis ('crush syndrome')" (*Brit. med. Bull.* 1945, **3**, 107); and "Aetiological factors in rheumatic heart disease" (*Brit. med. Bull.* 1952, **8**, 343).

DR A. H. CAMPBELL is an executive director of Glaxo Laboratories Ltd., formerly in charge of production and latterly of research on antibiotics. After graduating in botany at Edinburgh in 1931, he was appointed Assistant Lecturer in Mycology, subsequently moving to Bristol to continue teaching and research in this field. Originally specializing in plant pathology, he worked, while on sabbatical leave, for the Colonial Office in East Africa, and still retains his interest as a director of the Murphy Chemical Company Ltd. An early concern with the problems of producing penicillin made him a consultant with Glaxo in 1943 and later General Manager of the first deep-culture penicillin factory to operate outside of the United States of America.

PROFESSOR J. W. CROFTON is at present Professor of Tuberculosis and Director of the Department of Tuberculosis and Diseases of the Respiratory System at the University of Edinburgh, and Honorary Consultant Physician of the South Eastern Regional Hospital Board (Scotland). From 1947 to 1951 he was Lecturer, and later Senior Lecturer, in Chest Diseases, at the Postgraduate Medical School of London, and also Physician at Hammersmith Hospital, London. From 1947 to 1949 he was a part-time member of the Medical Research Council's Tuberculosis Research Unit at the Brompton Hospital, London, where from 1949 to 1950 he was Senior Assistant of the Institute of Diseases of the Chest. Professor Crofton's clinical work was carried out in London at Hammersmith Hospital and at the Brompton Hospital, and his research work has been concerned with pneumonia, pulmonary eosinophilia, chronic bronchitis and the chemotherapy of tuberculosis. He is joint author of "Pneumonia in West London, 1949-1950" (*Brit. med. J.* 1951, **2**, 1368) and of "Pulmonary eosinophilia" (*Thorax*, 1952, **7**, 1), and author of the chapter on tuberculosis in *Textbook of medical treatment*, edited by D. M. Dunlop, Sir Stanley Davidson & S. Alstead (7th ed., 1958).

PROFESSOR R. CRUICKSHANK was appointed to the Chair of Bacteriology in Edinburgh University in 1958 after having held a similar post in the University of London at St Mary's Hospital Medical School from 1949. From 1949 to 1956 he was Deputy Principal of the Wright-Fleming Institute of Microbiology, London, and was appointed Principal in 1955 when Sir Alexander Fleming resigned. Earlier, Professor Cruickshank had been Director of a group laboratory in the London County Council Hospital and Medical Services (1937–45) and Director of the Central Public Health Laboratory, Colindale (1946–48). Early in his career, Professor Cruickshank directed his attention to the laboratory and epidemiological aspects of infectious diseases and this has remained his main interest. He was associated in pioneer work in the serological treatment of lobar pneumonia and in 1933 gave the Milroy Lectures in the Royal College of Physicians on pneumococcal infections. He has carried out numerous studies on hospital infection. As a member of various scientific committees of the Medical Research Council and the Ministry of Health, he has helped to organize and direct controlled trials of prophylactic vaccines and of chemotherapeutic substances in tuberculosis, whooping-cough, influenza, typhoid fever, and other infections. Professor Cruickshank's present interests are mainly in the fields of chemotherapy, immunology and epidemiology. Since the war Professor Cruickshank has made many tours abroad under the auspices of the World Health Organization, of the British Council and of the Rockefeller Foundation. Some of these visits have been advisory when his help was sought in the problems of infectious disease which followed the war in Europe. Professor Cruickshank was editor of *Control of the common fevers* (1942) and sectional editor of *Recent advances in clinical pathology* (2nd ed., 1951). He has recently published, in association with co-workers, "Family studies of respiratory infections" (*Brit. med. J.* 1958, **1**, 119).

DR A. ERIC DORMER was trained at St Bartholomew's Hospital, London, where, after qualifying in 1951, he held resident appointments and was later appointed to the Medical Unit. Working initially under Professor R. V. Christie and Dr G. W. Hayward, he became interested in bacterial endocarditis, and had the opportunity of following patients who had been studied as part of the Medical Research Council bacterial endocarditis trial, and of discussing associated bacteriological problems with Professor L. P. Garrod. Previous publications concerning this subject have been in the *British Medical Journal* (1954, **1**, 500, with H. W. Balme; 1956, **1**, 612, with H. W. Balme & L. Rawlings; 1958, **1**, 63). During the year 1957–58 Dr Dormer was Fellow in Medicine at the Johns Hopkins Hospital, Baltimore, USA. He has also been concerned with the study of oxalate metabolism, working with a group under the direction of Dr E. F. Scowen, and publishing a series of joint communications.

PROFESSOR D. M. DUNLOP has been Professor of Therapeutics and Clinical Medicine in the University of Edinburgh since 1936. He is the Senior Physician in the Royal Infirmary, Edinburgh, and is chiefly interested in diabetic and endocrinological problems and applied pharmacology. His previous appointments have included the following: Chairman of the British Pharmacopoeia Commission, from 1948 to 1958; Vice-Chairman of the South Eastern Regional Hospital Board (Scotland); member of the Secretary of State's Advisory Committee on Medical Research in Scotland; member of the Cohen Committee on the Classification of Proprietary Preparations; Sims Commonwealth Travelling Professor, 1951; Lumleian Lecturer to the Royal College of Physicians of London, 1954; and a member of the editorial board of the *Quarterly Journal of Medicine*. At present Professor Dunlop is a member of the Ministry of Health Committee on Medical and Nutritional Aspects of Food Policy, and of the Interdepartmental Committee on Drug Addiction; he is also a member of the Expert Advisory Panel on the International Pharmacopoeia and Pharmaceutical Preparations of the World Health Organization. His publications include: chapters in *Textbook of medical treatment*, edited jointly with Sir Stanley Davidson & S. Alstead (7th ed., 1958); with C. P. Stewart, *Clinical chemistry in practical medicine* (5th ed., 1958); with L. J. P. Duncan & J. D. Baird, "A clinical trial of BZ 55" (*Brit. med. J.* 1956, **2**, 433).

DR E. F. GALE is Reader in Chemical Microbiology in the University of Cambridge and Director of the Medical Research Council's Chemical Microbiology Research Unit at Cambridge. The work of the Unit has been largely concerned with the organization of microbial cells, the mechanisms of protein synthesis and the actions of antibiotics both in disorganizing the cell and in preventing protein synthesis. Dr Gale's own studies have centred during recent years on protein synthesis in staphylococci and the action of chloramphenicol thereon. Publications are mainly in the form of papers to scientific journals, but summaries and reviews of the general field will be found in *Advances in Protein Chemistry* (1953, **8**, 285); in his Leeuwenhoek Lecture to the Royal Society (*Proc. roy. Soc.* B, 1957, **146**, 166); and in the 1959 Ciba Lectures in Microbial Biochemistry at Rutgers

NOTES ON CONTRIBUTORS

University, New Jersey, published by Wiley & Sons, New York, under the title *Synthesis and organisation in the bacterial cell* (1959).

PROFESSOR LAWRENCE P. GARROD holds the University Chair of Bacteriology at St Bartholomew's Hospital, London. His main interest for over thirty years has been the destruction of bacteria by chemical means: before the advent of antibacterial chemotherapy his work on germicides included devising a new technique for the Chick-Martin test which is now a standard method, an investigation of urinary antiseptics, and a study of a new series of acridine compounds in collaboration with A. Albert. He was an original member of the Penicillin Clinical Trials Committee of the Medical Research Council, which in March 1943 initiated the first studies of the therapeutic action of penicillin to be made outside Oxford, and he has served on similar committees since; he is Chairman of the present Antibiotics Clinical Trials Committee. His own studies of antibiotics include observations on the nature of their action in vitro and on the susceptibility to them of several imperfectly studied genera, comparisons of the activity of antibiotics in related groups, investigations into acquired resistance, and many observations on the clinical usefulness and limitations of antibiotics made in collaboration with clinical colleagues. Professor Garrod is Consultant in Antibiotics to the Army, and in 1958 was Lister Fellow of the Royal College of Physicians of Edinburgh, choosing as his subject in this connexion the chemotherapy of urinary tract infections. For seven years (1951–57) he was editor of the *British Journal of Experimental Pathology*.

DR J. C. GOULD is Senior Lecturer in Bacteriology at Edinburgh University, having previously been Senior Assistant Bacteriologist to the Royal Infirmary, Edinburgh. He has carried out research on virulence enhancement factors, the antibiotics, and infections due to staphylococci and *Pseudomonas pyocyanea*. Currently, he is carrying out investigations on hospital infections and on the distribution of antibiotics in the environment. Recent publications include: with W. S. A. Allan, "*Staphylococcus pyogenes* cross-infection" (*Lancet*, 1954, **2**, 988); with J. D. Cruikshank, "Staphylococcal infection in general practice" (*Lancet*, 1957, **2**, 1157); and "Environmental penicillin and penicillin-resistant *Staphylococcus aureus*" (*Lancet*, 1958, **1**, 489).

DR B. W. LACEY has been Reader in Bacteriology at Westminster Medical School, University of London, since 1952 and Bacteriologist to Westminster Hospital since 1947. He began laboratory work in 1937 as an assistant in the late Sir Lionel Whitby's laboratory at the Bland Sutton Institute, Middlesex Hospital, and joined the Army Blood Transfusion Service with him in September 1939. From 1942 to 1946 he served in the Royal Army Medical Corps as Pathologist to hospitals in the Middle East, Officer Commanding the Central Pathological Laboratory in Cairo and Assistant Director of Pathology, General Headquarters, Middle East Forces. His chief research interest is in the interaction of environment and microbial activity, particularly of the *Bordetella* (pertussis) group, and he has introduced a new medium and method for the cultural diagnosis of whooping-cough (Broadsheet No. 17 of the Association of Clinical Pathologists). At present he is Honorary Meetings Secretary of the Society for General Microbiology.

DR E. J. L. LOWBURY has for the past ten years been bacteriologist to the Medical Research Council's Industrial Injuries and Burns Research Unit at the Birmingham Accident Hospital. He is an honorary research fellow in experimental pathology at Birmingham University, and a member of the Medical Research Council's Committees on Antibiotics Clinical Trials and on Control of Cross Infection. During the war he worked for the Emergency Public Health Laboratory Service and later served as Specialist in Pathology with the Royal Army Medical Corps. At present he is working on various aspects of wound infection, with particular interest in burns. Dr Lowbury's publications include: with D. M. Jackson & E. Topley, "*Pseudomonas pyocyanea* in burns: its rôle as a pathogen, and the value of local polymyxin therapy" (*Lancet*, 1951, **2**, 137); "Cross-infection of wounds with antibiotic-resistant organisms" (*Brit. med. J.* 1955, **1**, 985); and, with H. A. Lilly, "The sources of hospital infection of wounds with *Clostridium welchii*" (*J. Hyg., Camb.* 1958, **56**, 169). He is a contributor to *Textbook of British surgery*, edited by Sir Henry Souttar & J. C. Goligher (1958).

DR JAMES MCC. MURDOCH is Senior Registrar in Medicine in the Royal Infirmary, Edinburgh. He qualified in 1944 and after military service in India returned to work in various teaching and non-teaching hospitals in the west of Scotland. Since 1953 he has worked in several Edinburgh hospitals and is a member of the clinical teaching staff of the University of Edinburgh. During the past six years he has been engaged in the study of the effects of antibiotic and chemotherapeutic compounds in the treatment of tuberculosis and in chronic bronchial infections. At present he is undertaking long-term research into the pathogenesis and treatment of chronic pyelonephritis. His publications include: with I. W. B. Grant, "Pulmonary tuberculosis due to bacilli resistant to streptomycin and isoniazid" (*Lancet*, 1955, **2**, 587); with S. M. Stewart, "Viomycin with oxytetracycline in the treatment of pulmonary tuberculosis" (*Brit. J. Tuberc.* 1956, **50**, 85); and, with A. H. Ioannidis, "Chloramphenicol in treatment of acute respiratory infection" (*Brit. med. J.* 1957, **1**, 1157).

DR G. G. F. NEWTON has been, since 1947, a member of the staff of the Sir William Dunn School of Pathology, Oxford, and since 1953 a member of the External Scientific Staff of the Medical Research Council. His work, which has mainly been concerned with chemical and biochemical aspects of antibiotics, is chiefly published in the *Biochemical Journal*. He contributed to the isolation and chemistry of the polypeptide antibiotic, bacitracin, and cephalosporin N, a new type of penicillin. Dr Newton is at present working on the chemistry of cephalosporin C, a close relative of the penicillin family of antibiotics that is resistant to the action of penicillinase, and on the isolation of the polypeptide hormone secretin.

DR M. R. POLLOCK has been a member of the Scientific Staff of the Medical Research Council since 1945, first under Sir Paul Fildes at the Lister Institute of Preventive Medicine, London, and, since 1949, as Head of the Division of Bacterial Physiology at the National Institute for Medical Research, Mill Hill, London. Previously he had studied medicine at Cambridge University and University College Hospital, London, and after two house appointments served in the Emergency Public Health Laboratory Service and in the Medical Research Council's Jaundice Research Unit during the war. Dr Pollock's main research has concerned the induced synthesis of enzymes by bacteria (in particular penicillinase) and he has also published work on the role of fatty acids in the growth of *Haemophilus pertussis* and other micro-organisms. Since 1958 he has been Secretary of the Medical Research Council's Committee on Chemotherapy.

PROFESSOR J. M. ROBSON holds the Chair of Pharmacology at Guy's Hospital Medical School, University of London, and was previously Lecturer in Pharmacology at the University of Edinburgh. He has published many papers on the pharmacology and physiology of sex hormones. During the Second World War he was engaged in research on toxic gases and was responsible, in collaboration with Dr C. Auerbach, for the discovery of the mutagenic action of mustard gas and other substances. Professor Robson's investigations on experimental corneal ulcers led to the introduction of sodium sulphacetamide in ocular infections. In addition to work on sex hormones, he is actively engaged in research on the chemotherapy of tuberculosis and leprosy. Amongst his publications are: *Recent advances in sex and reproductive physiology* (3rd ed., 1947); and, in collaboration with Professor C. A. Keele, *Recent advances in pharmacology* (2nd ed., 1956).

DR E. F. SCOWEN is Director of the Medical Professorial Unit, St Bartholomew's Hospital, London, and Reader in Medicine, University of London. Previous to this appointment he had been Second Assistant, First Assistant, and Assistant Director of the Medical Professorial Unit at that hospital and had worked with Dr Philip Smith in New York whilst holding a Rockefeller Research Fellowship. The scientific work has been largely concerned with endocrinology and its application to therapeutics. In recent years this has been largely in relation to the control of endocrine-dependent cancer, with particular reference to cancer of the breast, and a number of papers have been published on this subject, particularly in collaboration with other workers in the Imperial Cancer Research Fund.

MR GERARD W. TAYLOR has been Reader in Surgery in the University of London since 1955 and is Surgeon to St Bartholomew's Hospital, London, and Assistant Director of the Surgical Professorial Unit. In 1951 he was appointed Surgical Research Fellow at Stanford University, San Francisco, and undertook haemodynamic studies of the peripheral and pulmonary circulation. His main research interests have been associated with vascular surgery and particularly with lymphoedema. Current work includes the measurement of oxygen tension in tissue in peripheral vascular disease and clinical and experimental studies on wound infection. Among his publications are: with E. Holman, "Problems in the dynamics of blood flow: II. Pressure relations at site of an arteriovenous fistula" (*Angiology*, 1952, **3**, 415); "Lymphoedema" (*Post Grad. med. J.* 1959, **35**, 2); and, with R. A. Shooter, G. Ellis & Sir J. Paterson Ross, "Post-operative wound infection" (*Surg. Gynec. Obstet.* 1956, **103**, 257). Mr Taylor is a member of the Surgical Research Society, the Association of Surgeons of Great Britain and Ireland and other medical societies.

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Printed in Great Britain by Spottiswoode, Ballantyne & Co. Ltd., London & Colchester

BRITISH MEDICAL BULLETIN
VOLUME 16 NUMBER 2 MAY 1960

Scientific Editor: *N. B. Myant*

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The Thyroid Gland: Introduction

During the past ten years research on the thyroid gland has developed in many directions and to a much greater extent than might have been foreseen; for this reason the time has seemed ripe for a review in the *British Medical Bulletin* of the aspects in which advances have been most striking, and it is appropriate that the review should be published in the year in which the Fourth International Goitre Conference is to be held in London.

So far as the biochemistry of the thyroid gland is concerned, the most conspicuous advances have been made by the application of new techniques of chromatography and by means of tracer studies using radioactive isotopes; there is indeed no field of biochemical research in which the combination of these methods has proved more illuminating, partly owing, of course, to the convenient properties of the isotope ^{131}I that has mostly been used. Without the refinements of separation and detection of closely similar compounds that the new techniques have made possible, it is difficult to believe that the natural occurrence of tri-iodothyronine could

have been discovered, and this discovery has profoundly influenced thought on the nature of the active secretion of the thyroid gland; whilst thyroxine stands unchallenged as the principal thyroid hormone, we now think of the active secretion of the gland not as a single chemical substance but in terms of a family of iodine-containing amino acids closely related to thyroxine, which is itself by far the most abundant but not intrinsically the most active. Furthermore we can now feel confident that we understand the chemistry of the biosynthesis of the thyroid hormones, even though we still have to admit ignorance of the nature of the first essential biochemical event on which the process depends, namely the specific trapping and concentration of iodide by the thyroid gland.

The efficiency of the transport of the thyroid hormones in the blood and the proper regulation of the access of the hormones to their sites of action in the tissues are matters of just as great importance to the maintenance of normal physiological conditions as is the production of adequate amounts of the active compounds in the thyroid gland itself. Much progress has been made by the study of the proteins in the blood plasma that are capable of binding thyroid hormones and of the factors that influence the binding. The results obtained go far to explain the way in which thyroid hormones are carried from gland to tissue; they are also clearly relevant to such questions as transplacental transfer of hormones and the regulation of the access of the hormones to the tissues; beyond this point, however, we are still in the realms of speculation, for we do not know how the hormones gain access to their sites of action within the cells nor, indeed, in spite of much work at the subcellular level, can we identify the sites of action themselves with confidence. These questions cry out for solution but the effective approach has yet to be found.

So far as disease of the thyroid gland is concerned, the outstanding event is the revolution in the treatment of hyperthyroidism. The discovery of effective antithyroid drugs was itself a great advance, but its importance has been overshadowed by the application of radioactive isotopes of iodine. The use of these isotopes, first in trace amounts for the assessment of thyroid function and secondly in larger doses for the destruction of the desired amount of hyperfunctioning thyroid tissue, gives a measure of precise therapeutic control that is truly astonishing. Nevertheless, although the treatment of Graves's disease has thus been so greatly advanced, the aetiology of the condition remains unexplained. For this reason it is encouraging that more knowledge is being gained of the thyrotrophic hormone of the anterior pituitary, and even more that indications are now becoming available of the mode of control of the anterior pituitary itself by the hypothalamus. It seems most likely that the still vexed question as to whether or not Graves's disease is an autonomous disorder of the thyroid gland will ultimately be solved by further pursuit of the experiments by which the mechanism of the control of thyroid secretion is being traced further into the central nervous system. Even if the problem of the aetiology of Graves's disease is not directly solved by studies of this kind, they cannot fail to throw much needed light on the functional relationship of the central nervous system and the thyroid gland. The complementary approach based on the investigation of the much less understood actions of thyroid hormones on the central nervous system is clearly desirable.

One of the most striking recent discoveries in the field of thyroid research is the demonstration of the formation in certain conditions of thyroid autoantibodies. This discovery is of interest not only in its relation to disorders of the thyroid gland itself, particularly Hashimoto's disease, but also on account of the general immunological considerations involved. It already appears that the quantitative measurement of such antibodies can be useful in the differential diagnosis of thyroid disease; apart from this practical application, however, the conditions of production and the properties of thyroid autoantibodies deserve further study, the results of which may well prove to have an importance extending far beyond the range of thyroid research.

A conspicuous feature of modern medical research has been the increasing interest in the role of genetic factors in disease. It is therefore not surprising that much attention has been and is being paid to the genetic aspects of disorders of the thyroid gland, especially those in which familial tendencies have been suggested, and these studies are now beginning to bear fruit. Thus the analysis of the biochemical

abnormalities in sporadic goitre and cretinism, and the familial investigation of the association of deafness with thyroid dysfunction, do indeed indicate clearly the importance of genetic factors in these conditions. The role of such factors in other thyroid disorders is less definitely established, but there are suggestions which make further study highly desirable.

The papers contained in this number of the Bulletin deal with all the aspects of research on the thyroid gland that have been mentioned, together with several others of a more specialized character. Perusal of the contributions by the general reader will leave him with the impression of a considerable achievement. This impression will indeed be correct, but the full intention of this symposium will not be realized unless it conveys the further picture of a field of work in which activity continues to be intense for the very reason that there remain so many unsolved problems.

Some of these outstanding problems have been indicated in this Introduction and they are indeed of formidable difficulty. The long history of work on the thyroid gland, however, contains many illustrations of the peculiar fascination that research on this subject has exercised on scientific workers who have been drawn into it from many different disciplines. There is no reason to doubt that this state of affairs will continue and that new approaches will be found which will make it possible to take up the remaining challenges, to the great advantage of biology and medicine.

C. R. Harington

Sir Charles Harington acted as Chairman of the committee that planned this symposium, and Dr N. B. Myant as its Scientific Editor. We are most indebted to them both for all the help they have given in its production.

THE FOURTH INTERNATIONAL GOITRE CONFERENCE

President: Sir Charles Harington, F.R.S.

This conference will be held 5–8 July 1960, at Church House, Westminster, London, S.W.1.

Honorary Secretary: Selwyn Taylor, D.M., F.R.C.S., Royal Society of Medicine, Dering Yard, 67 New Bond Street, London, W.1

THE ASSOCIATION OF DEAFNESS WITH THYROID DYSFUNCTION

W. R. TROTTER D.M. M.R.C.P.

Medical Unit
University College Hospital Medical School, London

1 Deaf-mutism in relation to endemic cretinism and goitre
  a Endemic cretinism
  b Endemic goitre
  c Pathology of endemic deaf-mutism
  d Effect of iodine prophylaxis
  e Aetiology of endemic deaf-mutism
2 Sporadic goitre with deaf-mutism (Pendred's syndrome)
  a Clinical observations
  b Biochemistry of the thyroid disorder
  c Otological observations
  d Genetics
  e Relation of Pendred's syndrome to endemic goitre with deaf-mutism
References

Deafness is encountered in three types of thyroid disorder: adult myxoedema; endemic cretinism; Pendred's syndrome.

I do not propose to discuss the first of these associations in any detail. Briefly, it can be said that some impairment of hearing occurs in about half the cases of adult myxoedema (32 of the 60 cases studied by the Myxoedema Committee) (Clinical Society of London, 1888). In some of these the association is no doubt fortuitous, since myxoedema usually occurs in the second half of the life-span, when deafness from other causes is common. Otological examination shows that deafness in myxoedema may be perceptive, conductive or mixed (Mann, 1931; Despons, 1954; Marquet, 1956; Howarth & Lloyd, 1956). It may be caused in part by a general diminution in cerebral activity; a myxoedematous infiltration of the middle ear has also been postulated to explain loss of conduction. In about half the cases hearing improves when the patient is treated with thyroid extract (Howarth & Lloyd, 1956).

The degree of deafness encountered in myxoedema is seldom severe, whereas in endemic cretinism and Pendred's syndrome the loss of hearing is usually nearly complete. This is one reason for thinking that hypothyroidism in postnatal life is not likely to be more than a minor factor in the causation of deafness in the last two disorders.

1. Deaf-Mutism in Relation to Endemic Cretinism and Goitre

a. Endemic Cretinism

Towards the end of the eighteenth century European authors began to describe a state of "degeneration" peculiar to the valleys running down from the Alps and Pyrenees. The condition acquired the name "cretinism"; the best of the early accounts is that by Fodéré (1800). The most striking characteristic of this condition was its rather strict geographical localization; and it was early remarked that in localities where cretinism was present most of the inhabitants had goitres. "Cretinism" was never strictly defined, but it is clear that the three salient features were: retarded bodily growth (often amounting to dwarfism), retarded mental development (often amounting to imbecility), and severe deafness, which, since it appeared in very early life, often resulted in failure to acquire the faculty of speech (deaf-mutism). A goitre was usually present, but its size bore no relation to the general condition; in fact it was often remarked that the worst cretins tended to have little or no palpable thyroid tissue. Typical examples of the Alpine cretins are well shown in illustrations in the Report of the Sardinian Royal Commission (1848), and also in the book by de Quervain & Wegelin (1936), which remains the most authoritative and comprehensive account of this condition.

Cretinism has subsequently been described in many other parts of the world (Hirsch, 1885; Kelly & Snedden, 1958), notably in the Himalayas (McCarrison, 1908; Stott & Gupta, 1934). It is, however, very obvious that all authors do not use the term in quite the same sense. What is agreed is that states of impaired mental and physical development are unduly prevalent in certain restricted geographical areas. These areas are usually—but not invariably—mountainous, and they are always areas where goitre is also unduly prevalent. One small focus of endemic cretinism has been described in Britain in the village of Chiselborough (Somerset). There Norris (1848) found 4 "perfect idiots", 17 "partial idiots" and 5 deaf-mutes; most of the rest of the inhabitants had goitres. An interesting feature of this endemic was its transience; for only 23 years later Norris informed Fagge (1871) that Chiselborough was then virtually free of cretinism.

It must be emphasized that writers on this type of cretinism are not necessarily describing a single clinical entity. Benda (1946), for instance, considered that many of the inhabitants of Swiss institutions for "cretins" would have been classified in the United States of America less specifically as "mental defectives". In contrast to the varied symptomatology of endemic (i.e., geographically determined) "cretinism" is the well-defined nature of the two sporadic syndromes to which the name of "cretinism"[1] has also been applied. These two syndromes have two features in common: they show no tendency to geographical localization, and all their clinical features are clearly the result of hypothyroidism.

Historically, the first of these two syndromes to be described was that which is now called "sporadic goitrous cretinism" (*Medical Times*, 1855). This syndrome, which has been much studied in recent years, notably by Stanbury & McGirr (1957), is clearly caused by a congenital defect in the enzymic equipment of the thyroid, and is inherited in a recessive manner. The second (and more familiar) of the two syndromes has been called athyreotic or non-goitrous sporadic cretinism. It is associated either with total failure of the thyroid to develop during foetal life, or with the presence of a tiny fragment of thyroid at the base of the tongue. In either case the fault is clearly embryological. Although occasional instances of familial occurrence have been recorded, the evidence that this syndrome has a genetic basis is slender.

In both types of sporadic cretinism there is clear evidence (biochemical or anatomical) of a severe thyroid defect; and the success of replacement therapy enables us to attribute all the manifestations of the disease to hypothyroidism alone.

[1] See McGirr, p. 113 of this number of the Bulletin.—Ed.

Because it also is called "cretinism" there has been a tendency among physicians in non-Alpine regions to assume that the endemic condition can also be attributed to hypothyroidism in the same straightforward and unequivocal way. Reference to the work of those with first-hand experience of endemic cretinism shows, however, that this is an unjustifiable assumption. The mere fact that many persons described as endemic cretins are of normal stature (without having received specific therapy) is proof that they cannot have been severely hypothyroid during the growing period. In recent times Costa and his colleagues (Costa, Mortara, Cottino, Pellerito & Dall 'Acqua, 1959) have studied endemic cretins from the valleys of northern Italy, and shown clearly that they differ in numerous respects from sporadic cretins. The most notable finding was that the sporadic cretins showed unequivocal biochemical evidence of hypothyroidism, whereas the endemic cretins for the most part did not. Costa's work naturally raises the question of the definition of endemic cretinism. He is obviously using the term in its original sense of a state of mental and physical maldevelopment restricted to certain mountainous areas. English and American authors, on the other hand, have assumed with Curling (1850) and Fagge (1871) that sporadic cretinism is the same disease as endemic cretinism; and now use "cretinism" as synonymous with "congenital hypothyroidism". The use of the same term in these different senses has led to some controversy (Costa, 1957; Stanbury & Querido, 1957).

De Quervain & Wegelin (1936) make it clear that hypothyroidism was present in some degree in the endemic cretins studied by them at Bern. In the most severe cases the thyroid was virtually functionless and most of the clinical manifestations could be attributed to lack of its hormone. Nevertheless these workers were forced to conclude that some of the manifestations could not be attributed to hypothyroidism. Amongst these the most striking was deaf-mutism, which, as they pointed out, is not a manifestation of the "pure" hypothyroidism seen in sporadic cretinism.

A rather similar picture emerges from recent studies by Raman & Beierwaltes (1959) on the inhabitants of a village in an endemic goitre area in north-east India. Of their sample of 48 persons, 21 were goitrous, 17 were deaf-mutes and 10 were mentally defective. Only one was recognized as a "cretin" (in the Anglo-American sense), but it seems certain that most Alpine writers would also have applied this term to the mental defectives and the deaf-mutes. The mean serum protein-bound iodine (PBI) and butanol-extractable iodine (BEI) levels of the whole group of 48 persons were low by USA standards, and suggested that many of the inhabitants might be mildly hypothyroid. However, the PBI and BEI levels of the deaf mutes were not significantly lower than those of the other inhabitants. These recent observations conform to the views of de Quervain & Wegelin (1936), if it is admitted that these authors would have described as endemic cretins many of the subjects studied by Raman & Beierwaltes (1959). Here we have a picture of a community in which many of the inhabitants had PBI and BEI levels compatible with mild hypothyroidism; yet in which there were also deaf-mutes who were not notably more deficient in thyroid hormone than those with normal hearing.

b. Endemic Goitre

In the preceding section deaf-mutism was regarded as a manifestation of the ill-defined condition known as "endemic cretinism". It is also possible to approach the matter in a different way, by discarding this term and attempting to correlate the phenomenon of deaf-mutism with the phenomenon of goitre. This approach enables us to make use of statistical data, since in most countries deaf-mutes are collected into institutions, and their numbers are therefore ascertainable and can be compared with the results of contemporary goitre surveys.

As a basis for comparison we may use the results of the very thorough survey by Stevenson & Cheeseman (1956) of congenital deafness in Northern Ireland. In this area, where neither goitre nor deafness is endemic, the prevalence of all forms of congenital deafness was 0.3 per 1,000 inhabitants. Similar levels have been observed in low-lying parts of Europe, and in the USA (Bloch, 1906). However, in Switzerland, Bloch (1906) and Hanhart (1938) have recorded much higher rates, even up to 20 per 1,000 in some villages. Deaf-mutism seems to have been about equally prevalent in some parts of the Himalayan foot-hills (McCarrison, 1908; Stott & Gupta, 1934); and in the Andean provinces of Peru and Argentina (Greenwald, 1957).

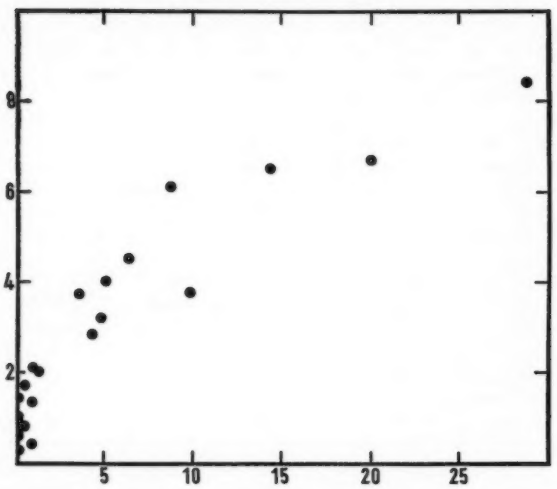
It will have been noted that the foregoing instances of endemic deaf-mutism have been associated with three great mountain ranges: Alps, Himalayas and Andes. These areas are also well known as centres of endemic goitre. In countries associated with these mountain ranges there seems to be a well-defined correlation between the prevalence of deaf-mutism and the prevalence of goitre. Stott & Gupta (1934), for instance, came to regard the occurrence of deaf-mutism in a region as indicating a severe type of endemic goitre. Census figures from Peru and Argentina (Greenwald, 1957) show a quite remarkable correlation between the prevalence of the two conditions in various provinces (see fig. 1). Less striking but still significant correlations can be found in similar figures from Switzerland and Italy.

In lowland areas, where the prevalence of both goitre and deaf-mutism is small, no correlation can be made out between the two conditions; Scotland is an example (Kelly & Snedden, 1958). Figures from the USA also show a fairly uniform distribution of deaf-mutism, with no tendency to rise in the mid-western "goitre-belt" adjacent to the Great Lakes. It is evident from a perusal of the comprehensive review by Kelly & Snedden (1958) on the prevalence and geographical distribution of endemic goitre (from which it would appear that the occurrence of cretinism and deaf-mutism is noted wherever it has been recorded) that cretinism and deaf-mutism are not a feature of all goitre endemics. Examples of areas where goitre is endemic, but deaf-mutism is not, are southern Tipperary in Ireland, Finland, El Salvador, Ethiopia and the Ruanda-Urundi area of the Belgian Congo (although deaf-mutism occurs in the about equally goitrous southern region of the Congo). These negative reports must be treated with considerable reserve, since deaf-mutes may well have been concealed by their families from the investigators. Nevertheless the prevailing impression is that some goitre endemics are accompanied by a high prevalence of deaf-mutism, while others are not.

It is noteworthy that, wherever deaf-mutism is recorded in connexion with a goitre endemic, cretinism is also said to be prevalent. This endorses the view of de Quervain & Wegelin (1936) that deaf-mutism can be regarded as a manifestation of endemic cretinism. However, at least one exception is known, for Secretan (1954) has recorded a high incidence of deaf-

FIG. 1. Prevalence of Goitre and Deaf-Mutism in 23 Provinces of Peru (Census of 1940)

(From the data of Greenwald (1957))



Abscissae: prevalence of goitre per 1,000 inhabitants
Ordinates: prevalence of deaf-mutism per 1,000 inhabitants

mutism (apparently genetically determined) in the Swiss village of Ayent, where cretinism does not occur and goitre is not endemic.

In detailed studies of an area in central Switzerland, Eugster (1938) has shown that there is a close correlation between the prevalence of goitre and the prevalence of endemic cretinism. Like de Quervain and Wegelin, he regarded deaf-mutism as an integral component of the syndrome of endemic cretinism. Gusic (1957) found abnormalities of cochleo-vestibular function in no less than 75% of persons with endemic goitre in the mountains of Montenegro.

c. Pathology of Endemic Deaf-Mutism

De Quervain & Wegelin (1936) have summarized what is known of the morbid anatomy of deaf-mutism, considered as a component of endemic cretinism. The main changes are found in the middle ear. The lining of the tympanic cavity is grossly thickened, leading to narrowing of the round and oval windows. The ossicles are also enlarged and distorted. The inner ear is also sometimes affected, the commonest anomaly being the occurrence of a hyaline ledge between the organ of Corti and the membrane of Corti.

Albrecht (1934) and Lüscher (1952) have given a similar account of the anatomical changes found in endemic deaf-mutism. Lüscher commented on the apparent discrepancy between the pathological finding that the most marked changes occur in the middle ear, and the clinical observation that in such cases the deafness is of the type associated with disease of the inner ear. De Quervain & Wegelin (1936) considered that the observed pathological changes probably started in late foetal life, but mainly took place during the first year of postnatal life.

From a study of the few recorded cases, de Quervain & Wegelin (1936) concluded that similar changes in the ear do not occur in "congenital athyreosis" (i.e., sporadic non-goitrous cretinism). Lüscher (1952) agreed with them that the abnormalities found in the endemic condition could not be attributed to hypothyroidism in postnatal life.

d. Effect of Iodine Prophylaxis

Wespi (1945) has made a detailed study of the incidence of deaf-mutism in relation to the introduction of iodized salt in the Swiss cantons. The incidence of deaf-mutism was calculated by arranging entries to schools for the deaf by year and place of birth. No attempt was made at an aetiological classification, and the recorded cases must therefore have contained a proportion of acquired deafness, caused by infections. He also had available figures for the proportion of all salt that had been iodized in each canton for each of the relevant years (the programme of iodization varied greatly from canton to canton, in both timing and extent).

The incidence of new cases of deaf-mutism in Switzerland as a whole was relatively constant between 1915 and 1922, at a level of 1.2–1.7 per 1,000 births. In 1923 it started to fall, and by 1925 had reached 0.4 per 1,000, a level found in most other European countries. After 1925 the curve remained in about this region. The curve for the proportion of salt iodized followed a very similar course. By 1925, 23% of all the salt used had been iodized. At first sight it seems difficult to attribute such an extremely large fall in the incidence of deaf-mutism to the iodization of a relatively small proportion of the country's salt. However, at that stage the extent of iodization varied greatly from one canton to another. Wespi was able to show that, among the cantons which had experienced the highest incidence of deaf-mutism, there was a positive correlation between the decline in deaf-mutism and the extent of iodization of salt. One apparent exception was the canton of Wallis, where the incidence of deaf-mutism remained high in spite of fairly complete iodization. However, this canton is known to contain large numbers of cases of hereditary deafness of a type not associated with cretinism or goitre. The canton of Bern presented the reverse anomaly, for there deaf-mutism had fallen from 1.7 per 1,000 in 1916–20 to 0.5 per 1,000 in 1926–30, although only 4–6% of the salt in the canton was iodized. Wespi did not comment on this apparent exception.

There is no doubt that Wespi has demonstrated a correlation in time between the decline of deaf-mutism and the introduction of iodine prophylaxis. Such a correlation, although suggestive, does not by any means prove a causal connexion. The period when the iodization programme was getting under way was one of active social development. Food was coming from far more varied sources, hygiene was being improved and people were moving about more and also tending to marry outside their own communities. It is impossible to exclude the possibility that one or more factors of this type may have been responsible for the decline in deaf-mutism rather than the iodization programme itself. It should also be remembered that considerable cyclical variations in the prevalence of deaf-mutism have been recorded, both in Switzerland and in South American countries, long before the prophylactic use of iodine was proposed.

e. Aetiology of Endemic Deaf-Mutism

The type of deaf-mutism under discussion does not occur in isolation, but is part of the clinical picture of "endemic cretinism". This condition is quite clearly associated in some

way with abnormality of the thyroid gland since (i) it is only found in areas where goitre is very prevalent, and (ii) part of the clinical features of endemic cretinism can be attributed to hypothyroidism.

Nevertheless deaf-mutism itself cannot be the result of hypothyroidism operating during postnatal life, since it does not occur in sporadic cretins. It could, however, be the result of hypothyroidism operating during foetal life. In this connexion we may recall the observation made by most writers on endemic cretinism, from Fodéré (1800) onwards, that the mothers of endemic cretins are nearly always goitrous, though not usually themselves cretins. From what we now know of iodine metabolism it seems clear that the goitre of a pregnant woman on a diet low in iodine would remove almost the whole of her meagre iodide intake, leaving little or none for the foetus. The foetus might therefore be so starved of iodine as to render it hypothyroid, and this might interfere with the development of the auditory system. Another possibility is that the damage to the auditory apparatus is caused by an unidentified "Kropfnoxe", the action of which could be counteracted, as de Quervain & Wegelin (1936) suggest, by minute quantities of iodine.

These theories all attribute endemic cretinism (including deaf-mutism) to environmental factors. An alternative view which has often been advocated (most recently by Clements (1958)) is that endemic cretinism may be largely determined by genetic factors. Clements pointed out that the condition has always been reported from relatively isolated communities where consanguineous marriages are inevitably common. He therefore suggested that endemic cretinism might be due to the operation of a recessive Mendelian factor, which had become "concentrated" in these remote communities. Clements does not explain, however, how this genic concentration should come to have such a marked geographical localization. The communities of the Alpine and Himalayan valleys may well have been isolated; but so have been (and still are) many other communities in non-mountainous districts, where endemic cretinism does not exist. According to modern genetic doctrines it would seem that the hypothesis that endemic cretinism is caused by a recessive Mendelian factor almost necessarily implies some heterozygote advantage. This can hardly be related to adaptation to high altitudes, since the distribution of cretinism has an upper, as well as a lower, limit in terms of height above sea-level (Hirsch, 1885; de Quervain & Wegelin, 1936). Another possibility is that heterozygotes are better able to adapt themselves to conditions of iodine deficiency, perhaps because they are more readily capable of forming large goitres; this would account for the almost invariable occurrence of goitre in the mothers of cretins. It would of course be possible to suppose that, outside the main endemic areas, a chance aggregation of recessive genes might cause a small and transient endemic, such as that at Chiselborough (Norris, 1848).

The most important observations on the inheritance of endemic cretinism are those of Eugster (1938); he includes deaf-mutism as an integral part of the syndrome. His studies were made in the cantons of Aargau and Zürich (Switzerland), where the over-all prevalence of endemic cretinism was about 6%. They revealed no evidence that genetic factors were of any importance in the causation of endemic cretinism. Eugster made the following points: (i) the prevalence of consanguinity in the parents of cretins was no greater than in the parents of non-cretins; (ii) the proportion of cretinous children was no greater if one parent were a cretin than if both parents were healthy; (iii) in five of the families studied, both parents were cretinous, yet many of their children were normal; (iv) of 9 pairs of uniovular twins, 6 were concordant with respect to cretinism, compared with 10 of 15 pairs of binovular twins (the results were essentially the same with respect to deafness alone). In a follow-up of one of Eugster's cases, Dieterle (1952) has described a striking example of an incestuous union between a brother and sister, both of whom were endemic cretins. The child of this union had been under careful observation up to the age of 16. His development was completely normal, and detailed investigation failed to reveal any suggestion of cretinism. This case by itself is almost enough to refute the idea that endemic cretinism in Switzerland is an inherited condition.

Clements' hypothesis obviously cannot apply to the endemic cretins investigated by Eugster. It would, however, be unwise to assume that endemic cretinism is a unitary disease; throughout the world genetic factors may contribute to its aetiology in other countries. The other suggestion by Clements (1958) —that outbreaks of rubella in adults, occurring in isolated unimmunized communities, might be partly responsible for endemic deaf-mutism—fails to explain why these outbreaks should have affected only those particular isolated communities in which goitre was also endemic. The balance of evidence favours the view that endemic cretinism (and the deaf-mutism which often accompanies it) is in the main the result of environmental rather than genetic factors.

2. Sporadic Goitre with Deaf-Mutism (Pendred's Syndrome)

a. Clinical Observations

In areas where most of the inhabitants have goitres it is necessarily difficult to disentangle the relationship between this condition and other abnormalities, such as deaf-mutism. In many ways this type of relationship is best studied where both conditions are relatively rare, so that the occurrence of goitre and deafness in the same individual is less likely to be fortuitous. Even in Britain, however, where the prevalence of both goitre and congenital deafness is relatively low, the presence of the two conditions in the same patient would not necessarily suggest an aetiological connexion, unless other members of his family were similarly affected. It was the observation of two sisters, both deaf-mutes and both with large goitres, that led Vaughan Pendred (1896) to publish what is probably the first account of this syndrome. He was practising at the time in Durham, a county in which (apart from Weardale) goitre has not been common (Stocks, 1928). The two affected sisters were members of a sibship of ten. Both were deaf from infancy and could not speak coherently; and had had goitres since the age of 13. One sister was an imbecile, but the other is described as very intelligent. Bodily development does not appear to have been abnormal. The parents and other sibs were healthy.

These features of what we might, for convenience, call "Pendred's syndrome" were also present in the 12 persons described by Brain (1927). These came from five families, all resident in the eastern part of London or adjoining areas. All 12 persons were deaf from birth and had goitres usually dating from middle childhood. There were no instances of either goitre or deafness occurring separately. Only single generations were affected. The physical and mental development of the affected persons appeared to be normal. Brain

concluded that a gene defect, inherited in a recessive manner, was responsible both for the deafness and for the error of thyroid metabolism which manifested itself as goitre. These conclusions have been confirmed by subsequent work.

Since the paper by Brain (1927), single families, in which two or more members showed Pendred's syndrome, have been described by Deraemaeker (1956) from Antwerp, Thieme (1957) from Michigan, Elman (1958) from Michigan, and Morgans & Trotter (1958) from London. McGirr, Hutchison & Clement (1959) studied five cases in Glasgow, and Fraser, Morgans & Trotter (1960) 28 members of 18 London families. Johnsen (1957) has also published an interesting account of two intermarried Danish families, among which there were 12 persons with the features of Pendred's syndrome, but who were also hypothyroid. It is becoming apparent that cases of this syndrome are readily discovered once its existence has become recognized; since our joint paper was completed, Dr Fraser has discovered about 30 additional cases.

Further experience has also shown that the deafness, though always dating from infancy, need not necessarily be complete. Similarly, the degree of thyroid enlargement can vary from a goitre weighing nearly 200 g. to a gland which is only just detectably larger than normal. The constant feature of the syndrome as at present defined is the association of some degree of congenital deafness with a demonstrable thyroid abnormality.

Pendred's syndrome occurs with about equal frequency in the two sexes (see Table I). In this respect it differs sharply from most other types of goitre, in which a large preponderance of females is usually observed, but resembles endemic cretinism (de Quervain & Wegelin, 1936).

b. Biochemistry of the Thyroid Disorder

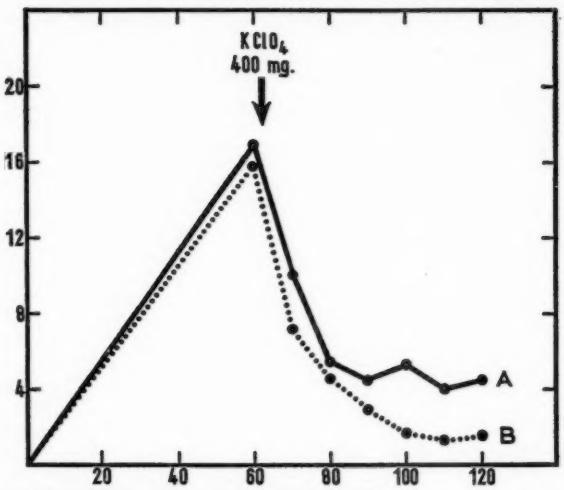
Thyroid function has been studied with radioactive iodine by Thieme (1957), Morgans & Trotter (1958), McGirr *et al.* (1959) and Fraser *et al.* (1960). Uptake of labelled iodine by the thyroid was either high or normal, except in one patient with a low uptake following partial thyroidectomy, recorded by Fraser *et al.* (1960). In some of the cases of McGirr *et al.* (1959), the initial clearance of radioiodine by the thyroid was very rapid. These observations suggest that the gland may be hyperplastic, as indeed it often is histologically (Smith, 1960), but tell us nothing about the nature of the causative lesion.

TABLE I. Proportion of Males and Females Affected in the Families Studied by Fraser, Morgans & Trotter (1960)

	Males	Females
Unaffected	16	15
Affected	10	18
Affected, less propositi*	4	6
Corrected ratio of affected to total: 10:41		

* Propositus: a modern adaptation of the participle of *proponere*, used for the first of a family to show some condition which on investigation proves to be familial.—ED.

FIG. 2. Discharge of Radioiodine from the Thyroid of a Patient with Pendred's Syndrome, following Oral Administration of Potassium Perchlorate



Abscissae: minutes after administration of ^{132}I
Ordinates: percentage of dose in thyroid
Curve A: results in untreated patient
Curve B: results after 100 mg. methimazole had been given one hour before the ^{132}I

The findings of Morgans & Trotter (1958), confirmed by McGirr *et al.* (1959) and Fraser *et al.* (1960) were more specific. When potassium perchlorate was given an hour or so after a tracer dose of radioiodine, the counting rate of the thyroid fell abruptly for a time, and then flattened out at a lower level (fig. 2). This suggests that a proportion of the labelled iodine held in the gland was at that time in the form of inorganic iodide. In normal subjects, and in patients with simple goitre or thyrotoxicosis, this discharge of radioiodine from the thyroid does not occur after the administration of perchlorate. However, if an incompletely effective dose of one of the thiouracil drugs has been given beforehand to any type of subject, perchlorate then has just the same effect as in the untreated patients with Pendred's syndrome. It may therefore be inferred that in these patients' thyroids there exists a partial block to the organic binding of iodine, without any impairment of the iodide-concentrating mechanism. The block is evidently only partial, for most of the patients were clinically euthyroid, and McGirr *et al.* (1959) and Fraser *et al.* (1960) were able to show chromatographically that both iodotyrosines and thyroxine were present in the excised glands of some cases. In the case illustrated in fig. 2, a more complete block could be induced by previous administration of methimazole, although the patient was partially hypothyroid. This shows that the naturally occurring iodination defect is seldom complete.

The ability of perchlorate to discharge labelled iodine from the thyroid clearly differentiates Pendred's syndrome from the general run of cases of simple goitre. However, the same phenomenon is also seen in certain rarer types of goitre. It is particularly well marked in the type of goitrous cretin originally described by Stanbury & Hedge (1950), examples of which have been studied also by Stanbury (1951), Lelong,

Joseph, Canlorbe, Job & Plainfosse (1956) and Schultz, Flink, Kennedy & Zieve (1957). In these cases the discharge of radioiodine was more complete than in the patients with Pendred's syndrome. In the descriptions of these cretins auditory acuity is not specifically mentioned, but it is of course often difficult to detect deafness in the presence of the severe degrees of mental defect manifested by some of these patients. Jackson (1954) described a case of this type, but the later studies of Fraser *et al.* (1960) showed that both the patient and her parents were examples of Pendred's syndrome. Clayton, Smith & Leiser (1958) described a negro family in which four of six sibs had goitres with defective organic binding of iodine; they were euthyroid and not obviously deaf. However, if audiograms had been done on their cases it is possible that a subclinical high-tone deafness might have been revealed.

The only non-familial condition in which a similar defect in the organic binding of iodine has been observed is Hashimoto's thyroiditis (Morgans & Trotter, 1957). Cases of Pendred's syndrome, however, do not show any of the serological (Fraser *et al.* 1960) or histological (Smith, 1960) features of this condition. The cause of the defect in organic binding is probably quite distinct in the two conditions.

The immediate cause of the inadequate binding of iodine which can be demonstrated in Pendred's syndrome could be a congenital deficiency of the enzyme (probably peroxidase) responsible for this step in thyroxine synthesis. Alternatively, the metabolic fault could lie outside the thyroid altogether, and achieve its effects on the gland by releasing into the circulation an abnormal metabolite with a thiouracil-like action. In an attempt to test the second hypothesis, we asked Dr J. Wolff (at that time working at the National Institute for Medical Research, London) to examine the effect of serum from some of our cases of Pendred's syndrome on the uptake and binding of labelled iodine by isolated slices of sheep and rabbit thyroid. The results were completely negative, although serum from the same patients after administration of a single dose of methimazole produced a marked reduction in iodine uptake and an almost complete inhibition of organic binding by the slices. These experiments need to be repeated, preferably using slices of the thyroid of a person with Pendred's syndrome, incubated in his own serum; it is possible that the hypothetical antithyroid substance might show a marked species specificity. Nevertheless it can be said that present evidence favours the view that the goitre in Pendred's syndrome is a response to an intrinsic enzyme defect in the thyroid.

Although direct evidence is lacking, it is probably safe to assume that the immediate cause of the goitre is an increase in the level of circulating pituitary thyrotrophic hormone, in response to a fall in circulating thyroxine. The clinical facts accord well with this hypothesis. Almost all our patients with Pendred's syndrome who had undergone a partial thyroidectomy later developed recurrent goitres. The only ones in whom this did not happen were being treated with thyroid extract. Our patients have all received thyroxine after the diagnosis has been established. Goitres so treated have always shown a decrease in volume, unless they were grossly nodular to palpation.

c. Otological Observations

The pathological changes in the ear have not yet been described in any well-authenticated case of Pendred's syndrome.

The symmetry of the deafness, the equal loss by both bone and air conduction, and the absence of detectable middle ear lesions, point clearly to the conclusion that the loss of hearing in Pendred's syndrome is of the perceptive type. The audiograms of the affected persons have shown a consistently greater loss of hearing for high than for low tones. Although there are no gross signs of labyrinthine disturbance, some of our subjects have shown a defective response to caloric stimulation. It would seem fairly evident that the otological lesion must lie in either the auditory and labyrinthine sense organs, eighth nerve or central nervous system.

All but two of the cases of Pendred's syndrome studied by Fraser *et al.* (1960) were clinically euthyroid, with normal levels of PBI in the serum. Their normal stature and body proportions provided evidence that thyroid function had not been grossly abnormal during the growing period. In these cases deafness could not be attributed to hypothyroidism. The clear genetic association of deafness with a specific thyroid abnormality suggests rather that both defects are derived from the same abnormal gene. The mechanism which enables a single gene defect to modify both thyroid and auditory apparatus remains, however, mysterious.

d. Genetics

Johnsen (1957) and Fraser *et al.* (1960) have each recorded a family in which a marriage between two persons with Pendred's syndrome has produced one or more affected children (the child of one of these marriages had been previously studied by Jackson (1954)). In all other recorded families Pendred's syndrome has been confined to single generations. This fact in itself strongly suggests a recessive mode of inheritance.

Although the recorded proportion of affected to total sibs is somewhat high, Table I shows that when Fisher's correction (1934) for inadequate ascertainment is applied by removing the propositi, the ratio is not far from the theoretical value of 1:4 to be expected in a condition which is inherited in a strictly recessive manner. Further confirmation of this hypothesis would be afforded by the existence of an abnormally high consanguinity rate among the parents of patients with Pendred's syndrome. In fact, a single instance of parental consanguinity has been noted by Deraemaeker (1956) and by Fraser *et al.* (1960). This amounts to two first-cousin marriages among 25 recorded matings. Since nearly all the recorded cases have occurred in western European populations, it is fairly safe to assume that this is an excessive consanguinity rate and therefore supports the hypothesis of recessive inheritance.

Fraser *et al.* (1960) considered that Pendred's syndrome accounts for over 1% of genetically determined deafness. This was almost certainly an underestimate, since their ascertainment was far from complete. In the Danish series reported by Lindenov (1945) of 277 congenital deaf-mutes there were 18 subjects (6%) with goitres who might well be regarded as having Pendred's syndrome. In the North American series recorded by Hopkins & Guilder (1949) of 339 children with congenital deafness there were probably eight cases of Pendred's syndrome (2%). As all their subjects were children, this is probably an underestimate, since the goitre of Pendred's syndrome is not necessarily obvious until adult life. Even Lindenov's figure of 6% may not represent the true prevalence, since he was not specifically looking for goitres. Probably the best estimate we can reach at present

is that 5–10% of all cases of congenital deafness are examples of Pendred's syndrome.

Taking the prevalence of congenital deafness as 0.3 per 1,000, the frequency of Pendred's syndrome in the total population can be assessed as between 1.5 and 3.0 per 100,000 inhabitants.

Accepting the hypothesis of recessive inheritance, Fraser *et al.* (1960) considered that the frequency of the abnormal gene was between 1 in 150 and 1 in 500.

At the present time it is not possible to recognize heterozygotes for the abnormal gene. Perchlorate tests and audiograms of parents of subjects with Pendred's syndrome have not shown any characteristic features.

e. Relation of Pendred's Syndrome to Endemic Goitre with Deaf-Mutism

There are obvious resemblances between the sporadic and endemic syndromes. In both, a perceptive type of deafness starts in early life in subjects with a demonstrable thyroid abnormality. In both, hypothyroidism may occur, but is by no means inevitable; yet it seems difficult to accept hypothyroidism as a cause of the deafness in either (although hypothyroidism in foetal life is a possible factor in the endemic cases).

Yet when we examine what is known of the aetiological factors involved, differences emerge between the two conditions which seem at present almost irreconcilable. Available evidence points to some environmental phenomenon as the cause of endemic cretinism; whereas Pendred's syndrome has all the characteristics associated with a hereditary defect which is inherited in a recessive manner. We must therefore either accept that a remarkable coincidence has occurred, and two quite unrelated sets of factors happen to have brought about two independent syndromes, in both of which congenital perceptive deafness is associated with a thyroid defect; or we must seek some final common pathway which would enable genetic and environmental factors to lead to a similar end-result. It would not be difficult to postulate a circulating substance or substances, acquired in the endemic cases from the environment (the "Kropfnoxe" of de Quervain and Wegelin), and produced, in Pendred's syndrome, by an enzymic block anywhere in the body; and to suppose that this substance (or substances) had toxic effects both on the cochlea and on the thyroid. Some drugs can cause a perceptive type of deafness (e.g., quinine, streptomycin), while numerous substances, related and unrelated to thiouracil, can cause the type of iodination defect found in the thyroids of patients with Pendred's syndrome. There is no evidence at present to support the hypothesis that either endemic cretinism or Pendred's syndrome is caused by a circulating toxic substance; and the only merit of this hypothesis is to suggest that it would be worth inquiring in detail whether endemic goitre with deaf-mutism could be a phenocopy of Pendred's syndrome. In particular, we need to know whether cases of endemic goitre with deaf-mutism show the same response to perchlorate as do cases of Pendred's syndrome.

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TYPES OF THYROID GROWTH

I. DONIACH M.D.

Department of Pathology
Postgraduate Medical School of London

1 Compensatory hypertrophy
2 Goitrous hyperplasia
3 Reparative growth
4 Foetal differentiation
5 Infantile growth
6 Neoplasia
7 Conclusions
References

Compensatory hypertrophy of the thyroid gland after partial thyroidectomy, and the reconstitution of grafted thyroid tissue, were both demonstrated in experimental animals in the nineteenth century. Since then it has been shown that these two types of thyroid growth differ. Compensatory hypertrophy is dependent on secretion of thyrotrophic (thyroid-stimulating) hormone (TSH) by the anterior pituitary, but the initial proliferative stage of reconstitution of grafts can occur in animals whose TSH secretion is suppressed.[1] Other types of thyroid growth include goitrous, neoplastic, foetal and infantile. Many experiments on the thyroid during the past 20 years have been concerned with TSH effects and the mechanism of TSH control. It is perhaps not generally appreciated that certain types of non-neoplastic thyroid growth and differentiation appear to be independent of TSH. In the following paragraphs the various kinds of thyroid growth enumerated are briefly discussed from the view-point of TSH dependence.

1. Compensatory Hypertrophy

Removal of part of the thyroid gland leads to hypertrophy of the remnant (Horsley, 1886; Halsted, 1896). Histological examination shows increased vascularity, decrease in follicle diameter, reduction in colloid store and increase in size of follicle cells, owing to enlargement of both nuclei and cytoplasm. The degree of activity is proportional to the amount of thyroid removed. Subtotal thyroidectomy gives rise to mitotic proliferation of epithelial cells and the formation of new follicles. A similar hypertrophy and hyperplasia is produced in the intact gland by administration of TSH. Compensatory hypertrophy is prevented by thyroxine administration (Marine, 1932) which suppresses TSH secretion, and by hypophysectomy. As a result of compensatory hypertrophy induced by TSH, a normal output of thyroxine is maintained after partial thyroidectomy by increased turnover of iodine and reduction of the colloid store. The term compensatory hypertrophy implies response to an increased functional load. However, hypertrophy after partial removal of an organ may occur in the absence of secretory deficiency, as seen in the hypertrophy of the testis after unilateral orchidectomy in hypophysectomized animals (Edds, 1958). Though a similar effect was not detected in the thyroid (Logothetopoulos & Doniach, 1955), it is likely that a slight change might be missed. The bulk of the thyroid in hypophysectomized animals consists of colloid. After hemithyroidectomy, a slight reduction in colloid store and an increase in cell size would leave the weight of the residual lobe unchanged. If one postulates the occurrence of compensatory hypertrophy of the thyroid independent of TSH, it must be very slight in degree, but might be revealed by measurement of cell height in the thyroid remnant of hypophysectomized animals submitted to subtotal thyroidectomy.

2. Goitrous Hyperplasia

Working in an endemic goitre area, Marine & Lenhart (1909) found that compensatory hypertrophy after partial thyroidectomy was considerably reduced by administration of iodine. They also found that goitrous thyroids contained less than the normal quantity of iodine per unit mass of thyroid tissue. Marine postulated that the development of goitre is comparable to hypertrophy of the thyroid remnant seen after partial thyroidectomy and that goitre results from compensatory hypertrophy in response to iodine deficiency. This interpretation has proved correct and is also applicable to the goitrogenic action of antithyroid drugs, where the stimulus to increased TSH secretion is not iodine deficiency but inhibition of thyroxine synthesis. The goitrogenic action of antithyroid substances is prevented by hypophysectomy or thyroxine treatment (Griesbach, Kennedy & Purves, 1941; Astwood, Sullivan, Bissell & Tyslowitz, 1943). TSH output is controlled by the amount of circulating thyroid hormone; it is stimulated by a fall and inhibited by a rise in blood thyroxine. The levels of hormone at which this feed-back system operates are modified by the needs of the body. Thus, a fall in blood thyroxine will stimulate a smaller rise in TSH output in a hot environment than in the cold (Dempsey & Astwood, 1943). To date, all the examples seen of non-neoplastic thyroid growth response to endogenous TSH stimulation can be explained as attempts at increased synthesis and secretion of thyroid hormone.

3. Reparative Growth

The results of early experiments of thyroid grafting and their interpretation proved conflicting. This is not surprising, since many were complicated by sepsis and iodine deficiency, and the experimenters were unaware of the homograft problem. When one thyroid lobe of a rat is grafted under its own skin, most of the lobe undergoes ischaemic necrosis. A new small functioning thyroid lobe made up of normal follicles is reconstituted in about ten days from surviving peripheral follicles. The graft made in this way, known as an autograft, survives the lifetime of the animal, and retains the physiological properties of normally situated thyroid tissue, its activity reflecting the level of circulating TSH (Marine & Rosen, 1934). Thus, if the residual lobe in the neck is subsequently removed, the graft hypertrophies (Cristiani, 1903); it shrinks under thyroxine administration (Marine, 1932), but does not wither away.

During the first few days after implantation, the function of living follicles in the graft is reduced, judged by their diminished

[1] See Purves & Adams, p. 128 of this number of the Bulletin.—Ed.

uptake of radioactive iodine (Bennett & Gorbman, 1951). Histological examination shows mitotic proliferation of follicular epithelium whose cells are small, columnar and crowded together. The follicles become angular and bud off new follicles. This reparative phase is not prevented or inhibited by thyroxine in dosage sufficient to suppress TSH secretion (Dempster & Doniach, 1955). Thyroid autografts take successfully in hypophysectomized rats (Williams & Doniach, unpublished experiments, 1959). Thus, reparative growth appears to be independent of TSH. The resultant tissue responds normally to TSH stimulation but survives in its absence. Follicles of a "reparative" type may be seen in human thyroids in the parenchyma flanking a haemorrhage or adenoma and in subacute thyroiditis. Fifty years ago, a "successful" endocrine graft was a vascular hypertrophied one with histological features suggestive of secretory activity. Experiments were interpreted to show that the "success" of a graft was dependent on the establishment of gross deficiency of the endocrine tissue implanted. This concept is still misapplied today to the take and survival of grafts, though these were proved 30 years ago to be independent of specific hormonal deficiency (Loeb, 1930). The stimulus to reparative hyperplasia is bound up with local trauma or ischaemia or cell death. The resultant hyperplasia is self-limited. The mechanism and control of this type of thyroid growth independent of TSH is quite unknown. It is independent of the blood hormone level and of removal of thyroid tissue, since multiple thyroid grafts take in inbred animals with intact thyroid glands (Loeb, 1930).

4. Foetal Differentiation

The primitive foetal thyroid consists of closely packed plates of syncytial masses of cells. Differentiation occurs fairly sharply. Colloid droplets appear in the cell masses and are followed by the development of microfollicles. Follicular differentiation takes place on about the 11th day in the developing chick, 19th day in the rat, 22nd day in the rabbit and 12th week in man (Gorbman, 1955). The foetuses of rabbits (Jost, 1947) and rats (Wells, 1950) have been shown to survive decapitation and hence hypophysectomy. Jost (1953) found that hypophysectomy in the rabbit foetus, done at a stage when the thyroid is still mostly solid, does not prevent subsequent follicular differentiation, though this develops a little more slowly than in controls. Sethre & Wells (1951) found that developing rat thyroids undergo hypertrophy in response to injections of TSH given directly into the foetus at a stage when follicular differentiation is already established. Thus, follicular differentiation in the foetus proceeds in the absence of TSH. Once initiated, differentiation may be apparently speeded up by TSH to which the newly formed follicles are sensitive. The hyperplastic response of differentiated foetal thyroid tissue to endogenous TSH stimulation is similar to an adult's and is best seen with thiouracil treatment. The goitrogenic action on the foetus of thiouracil given to the mother is seen only after follicular differentiation of the foetal thyroid has occurred (Logothetopoulos & Scott, 1956). Maternal TSH does not cross the placental barrier; thiouracil does (Peterson & Young, 1952).

Carpenter (1942) and Gaillard (1954) found that follicular differentiation takes place in tissue culture. It occurred after 4 days' growth of solid thyroid from chick-embryo explanted at the 8-day stage. Gaillard (1954) noted that the follicle-lining cells were smaller than in 12-day embryos used as controls. The addition of TSH to the culture medium increased the number of colloid droplets but not the rate of follicular differentiation.

5. Infantile Growth

Hypophysectomy in young animals limits growth in general (Smith, 1930). Little experimental work on this subject has been reported with special reference to the thyroid, and none on new-born animals. In new-born rats the thyroid grows extremely rapidly during the first few weeks. The rapid growth is not associated with increased function. In fact, assays during the first week show a low level of iodide concentration by the thyroid (Williams and Doniach, unpublished experiments). This finding may be interpreted as evidence that TSH secretion is low and not essential to the rapid growth of the thyroid gland. Infantile thyroid growth remains to be studied, but results to date suggest that in the rat it may well be independent of TSH.

6. Neoplasia

Though a minority of spontaneous human thyroid carcinomata are functional and their growth is suppressed by thyroxine, the majority appear to be independent of TSH. This contrasts with the thyroid tumours induced in rats and mice by prolonged goitrogen treatment, most of which respond functionally to fluctuations in level of TSH (Bielschowsky, 1955). In addition to morphological differences from normal thyroid, and behaviour differences in metastasizing tumours, the neoplastic tissue shows a functional difference in that iodine is bound organically in the presence of enough goitrogen to inhibit organic binding of iodine in neighbouring normal non-neoplastic follicles (Money, Fitzgerald, Godwin & Rawson, 1953). Experimentally induced thyroid tumours are at first transplantable only into thyroxine-deficient hosts, confirming their TSH dependence. However, after serial transplantations, autonomous tumours have arisen that grow in normal hosts, some of them still able to bind iodine organically (Morris, Dalton & Green, 1951; Purves, Griesbach & Kennedy, 1951; Wollman, Scow & Morris, 1953). In these studies, TSH-dependent transplantable tumours have shown varying degrees of impairment of thyroxine synthesis. Thyroid neoplasms therefore show a wide spectrum in types of growth in relation to TSH dependence. In some cases their functional behaviour is similar to adult tissue, in some it resembles foetal thyroid and in others appears different from any other type. Autonomous neoplastic growth differs from reparative growth in that the latter is sharply self-limited. The mechanism whereby prolonged TSH-induced hyperplasia changes over to neoplasia is unknown. It may be related to the excessive mitotic proliferation induced in an adult organ whose cells very rarely divide under normal conditions (Doniach, 1958).

7. Conclusions

TSH secretion normally stimulates thyroid growth only in so far as compensatory hypertrophy is required by functional needs of thyroxine secretion. Though sensitive to TSH stimulation, reparative thyroid growth and follicular differentiation of the foetal thyroid occur in the absence of TSH and are

initiated and controlled by unknown factors. It is possible that infantile growth of the thyroid belongs to a similar TSH-independent category. The induction of thyroid neoplasms by prolonged TSH stimulation is thought to result from the prolonged hyperplasia rather than any direct carcinogenic action of TSH. The varying degrees of TSH dependence found in thyroid neoplasms reflect varying degrees of resemblance to non-neoplastic types of thyroid growth. Experimental findings show a rough correlation between loss of function and degree of autonomy in thyroid neoplasms.

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GENESIS OF THE THYROID NODULE

SELWYN TAYLOR D.M. M.Ch. F.R.C.S.

Surgeon
King's College Hospital, London
Belgrave Hospital for Children, London

Surgeon and Lecturer in Surgery
Postgraduate Medical School of London

1 Anatomy and blood supply
2 Effects of iodine lack
3 Autoradiographic studies
4 Experimental nodular goitre
References

The term "nodule" is a particularly apt one when discussing lesions arising in the thyroid gland, for it is impossible to draw any sharp differentiation between focal hyperplasia and benign or even malignant tumours in this organ. If by the term adenoma is meant a well-circumscribed, encapsulated tumour, with no evidence of invasion or metastasis (Willis, 1948), then most thyroid nodules are adenomata. It has recently been estimated that more than two hundred million people in the world are affected with simple goitre (Kelly & Snedden, 1958), and many of these glands harbour one or more nodules. The following account is concerned with the way in which these nodules arise.

1. Anatomy and Blood Supply

A study of the anatomy and blood supply of the human thyroid gland is helpful in interpreting the manner in which nodules appear. The unit of secretion in the gland is the follicle, a spherical structure composed of a single layer of cuboidal cells with a basement membrane, and covered with a rich network of capillaries. The lumen of the follicle is usually filled with a clear viscid substance, colloid, which in health contains stored thyroid hormones. Twenty to forty follicles are bound together with connective tissue to form a lobule, and groups of these lobules are incorporated into lobes. A single artery supplies each lobule, as has been demonstrated by Johnson (1953) who perfused excised thyroid glands with warm saline, causing the interlobular fascial planes to swell up with oedema. It is these groups of 20–40 follicles which have been shown to function together in the autoradiographic studies described below. The blood supply of the thyroid breaks up into a mass of anastomosing vessels on the surface of the gland and these then dip down in the interlobar septa to reach the lobules. Injection studies of these vessels were carried out as long ago as 1884 (Bégoune, 1884), and long-standing nodular goitres are frequently shown to have an enormous blood supply. Wangensteen (1929) described how, in the goitres of cretins seen in de Quervain's clinic in Bern, the inferior thyroid artery sometimes equalled the size of the common carotid. It could be postulated that nodules occur in the thyroid because blood is shunted to a localized area, and Modell (1933) described arteriovenous shunts in the dog's thyroid and demonstrated by special staining methods that there were little pads of tissue which could alter the direction of the blood flow. No such arrangement has been discovered in man.

2. Effects of Iodine Lack

The primary cause of simple goitre is failure of the thyroid gland to obtain sufficient iodine to maintain its normal structure and function. The mechanism by which a reduced intake of iodine produces thyroid hyperplasia has been largely investigated by means of animal experiments. It is believed that, with a diminished iodine intake, the level of circulating thyroid hormones (protein-bound iodine, PBI) is reduced. This decrease in the amount of circulating thyroid hormones stimulates the anterior pituitary to produce more thyroid-stimulating hormone (TSH), and this in turn leads to characteristic histological changes in the thyroid. There is an increase in the follicular cell height (Rawson & Starr, 1938) and in time an increase in the total number of follicles, thus leading to an enlarged and vascular thyroid gland. The accompanying histological changes in the anterior pituitary are also characteristic, with the eventual appearance of "thyroidectomy cells" and decreased acidophilia (Griesbach, 1941). In its extreme form these changes may lead to an over-all increase in the size of the pituitary—a fact reported over a hundred years ago by Nièpce, who described the large pituitaries he found in goitrous cretins at post-mortem (Nièpce, 1851).

Careful goitre surveys in the endemic areas of the world have resulted in a clear picture of the natural history of thyroid enlargement in man and animals (Olesen, 1924, 1926; Olesen & Taylor, 1927). In an area of average endemicity, surveys will usually show that the thyroid gland is moderately enlarged in early childhood—the type of enlargement that was called the Rossetti neck by the Medical Research Council's goitre survey in 1948 (Medical Research Council, 1948). At the age of five years, about a quarter of the boys and a third of the girls may be so affected and by puberty the incidence is usually at its highest. Indeed, puberty goitre is one of the more sensitive indices of iodine lack in a region and for this reason has often been used for research by public health workers in the past. McCarrison (1917) thought that puberty goitre was a physiological rather than a pathological response to iodine lack, because of the greatly increased demands made by the growing body. However, such changes appear not to be entirely reversible, as adults may be seen with nodules in the thyroid gland who spent only the first 5–10 years of their lives in an endemic goitre area and subsequently moved to a district where the intake of iodine was apparently adequate (Taylor, 1958). A further explanation for the increase in the size of goitre at puberty and during pregnancy is provided by Cassano, Baschiéri & Andreani (1959) who report an enhanced clearance of iodide by the kidney at these times. It is possible that this additional loss of iodide from the body explains the increase in thyroid hyperplasia. Following puberty, the thyroid gland frequently diminishes in size in the male, except in severely endemic areas, but in the female it persists, and enlarges a little before each menstrual period and considerably during pregnancy. At the age of 30, nodules have often appeared; the problem then is why the diffuse hyperplasia of puberty should progress to the multinodular goitre of the adult.

Although lack of iodine is the basic cause of goitre, a great many other factors may increase the severity of the condition when the intake of iodine is already low. Calcium has been known for a long time to increase the severity of goitre (Boussingault, 1831) and if weanling rats are fed on a diet low in iodine the addition of calcium carbonate is particularly goitrogenic (Taylor, 1954). This observation correlates well with the fact that in the areas of the world poor in iodine, where there is in addition much chalk or limestone, goitre is more common. Simple goitre in England used to be called Derbyshire neck, and the geological formation of that county is largely limestone which is deeply cut into by the rivers Derwent and Dove. Pollution of drinking-water with the excreta of human and animal origin has also been shown to increase the incidence of goitre in areas poor in iodine. The pioneer investigations of McCarrison (1906) first drew attention to this when he studied the incidence of goitre in the group of nine villages which make up Gilgit in the foot-hills of the Himalayas. These villages, situated one below another, drew from the same water supply, which became more and more polluted with excreta as it descended. McCarrison's survey showed that eight of the nine villages presented a steadily rising incidence of goitre directly proportional to the increasing pollution; the exception was the one village which had an independent water supply from a spring. McCarrison filtered the solid matter from the polluted water and gave this twice daily to himself and a group of army volunteers who had been drafted to the district. One-third of the volunteers, including McCarrison, developed goitre between the 10th and 15th day, the swelling reaching maximum size between the 25th and 30th day; all the goitres disappeared after the experiment. Although the iodine content of the water and food in this area was low, this did not in itself produce goitre; this was well demonstrated when a further group of volunteers was given the same polluted water after it had been boiled, for in none of them did a thyroid swelling appear. Some years after this experiment, Marine & Lenhart (1910a, 1910b) described an epidemic of goitre due to water pollution in a trout hatchery. The fish were reared in tanks situated one below another, the effluent from one tank running down into the next. The degree of thyroid hyperplasia increased with the degree of pollution.

Iatrogenic goitre may occur as a result of prolonged therapy with drugs which either block the iodide-trapping mechanism of the thyroid, such as thiocyanate (Wolff, Chaikoff, Taurog & Rubin, 1946) and perchlorate (Stanbury & Wyngaarden, 1952), or interfere with the binding of iodine to protein, such as *p*-aminosalicylic acid (PAS) (Hamilton, 1953), resorcinol (Bull & Fraser, 1950) and cobalt (Kriss, Carnes & Gross, 1955). Many foods[1] are also known to have goitrogenic properties, if only to a slight degree. With the extremely sensitive methods of studying thyroid function which the introduction of radioactive iodine permitted, Fertman & Curtis (1951) were able to present a detailed study of this property in a large number of commonly ingested foods. However, the one receiving most attention so far has been the cabbage, ever since Chesney in 1928 discovered that rabbits fed almost exclusively on it developed large goitres (Chesney, Clawson & Webster, 1928). Subsequently Hercus & Purves (1936) and Kennedy & Purves (1941) in New Zealand showed that rape-seed, which also belongs to the genus *Brassica*, was a more powerful goitrogen and this led to an appreciation of thiourea as a goitrogen (Kennedy, 1942) and to the subsequent introduction, by Astwood (1943), of thiouracil as a valuable therapeutic agent. The active principle in the genus *Brassica* was isolated by Greer, Ettlinger & Astwood (1949) and is a thiooxazolidone.

There are other factors which may lead to the production of nodular goitre, especially in the young, and probably the most important of these are those inborn errors of iodine metabolism described elsewhere in this Bulletin ("Sporadic goitrous cretinism", by E. M. McGirr, p. 113; "The association of deafness with thyroid dysfunction", by W. R. Trotter, p. 92).

3. Autoradiographic Studies

The use of autoradiography, that is, the exposure of a section of tissue to photographic emulsion so that the latter is blackened where radioactive material is present in the tissue, is of particular value in the study of goitre. It makes it possible to correlate the histological appearances of any area with its ability to concentrate and combine iodine with protein—usually an indication of hormone synthesis. More than two hundred patients have been investigated by this technique (Taylor, 1953), and the results are probably representative of most types of simple goitre; some were drawn from those with sporadic goitre in London and others from endemic areas, mostly in southern Ireland, who had come to work in London. Each patient was admitted to hospital and, after a thorough history had been obtained, including details of dietary habits, district where born and bred and any family incidence of goitre, was given a tracer dose of radioiodine. The urine was collected for 48 hours and the over-all activity of the gland determined by the method of Fraser, Hobson, Arnott & Emery (1953). Partial thyroidectomy was then performed. Slices of tissue were cut through whole lobes to get a representative picture and these were then prepared by routine histological methods and giant sections cut. These were placed in contact with *x*-ray film in a box excluding light, and subsequently the films were developed and compared with the sections which had been suitably stained.

Examination of these autoradiographs showed that in diffuse simple goitre or puberty goitre, the blackening was peppered fairly uniformly throughout the gland, but in older patients the greater part of the tissue showed little ability to concentrate radioactive iodine and there were only small discrete foci of blackening. Blackening takes place only in areas where there is iodine-containing colloid, and colloid does not necessarily contain iodine. The features which distinguished those follicles capable of concentrating iodine were that they were small in diameter, with tall cells. When the diameters of a hundred follicles in active and non-active areas were measured in two axes at right angles to each other, it was found that the diameters of the active follicles were only about a third of those of the inactive ones. In addition, the active ones were grouped together and were much more uniform in size (Taylor, 1952).

When the autoradiographs of these simple goitres were examined in conjunction with the histological preparations and the case histories of the patients, it appeared possible to reconstruct the natural history of the disease (Taylor, 1953). The evolution of nodular goitre was divided arbitrarily into

[1] But the amounts of these foods normally eaten by human beings are probably not large enough to cause goitre (see paper by Clements on p. 133 of this number of the Bulletin).—Ed.

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five stages, although it is agreed that other workers might interpret the results differently.

Stage 1. This was the typical puberty goitre, although it may persist for years. There was diffuse enlargement, increased vascularity, large uptake of radioiodine and fairly uniform blackening of the autoradiograph.

Stage 2. This included all those glands which showed diffuse enlargement, but only focal areas of activity. There were small, discrete patches of blackening on the autoradiograph. Occasionally a patient was found in whom all the functioning tissue was concentrated in a single area: a toxic nodule in a non-toxic gland (Rawson, personal communication).

Stage 3. The gland was nodular and the centre of the nodule had been destroyed by haemorrhage. That this was recent was shown by the fact that the autoradiograph showed activity in the undamaged outer shell of tissue.

Stage 4. This was the end-result of stage 3, the nodule having resolved in one of two ways: either being filled by a large lake of colloid which was usually free of radioiodine, or replaced by a mass of new follicles which did not take up radioiodine.

Stage 5. This was the multinodular or "pudding-stone" goitre which had been produced by the continual repetition of the processes described above. One or two nodules could usually be found which probably provided the body's requirements of thyroid hormone; the remainder of the nodules were inactive.

4. Experimental Nodular Goitre

The vast experiments in goitre production which Nature has perpetrated in the endemic areas of the world have stimulated research, such as McCarrison's in the Himalayas 60 years ago (McCarrison, 1906), and Stanbury's in Argentina more recently (Stanbury, Brownell, Riggs, Perinetti, Itoiz & Del Castillo, 1954); these great endemics can be imitated in miniature and carefully studied in the laboratory by giving animals a diet low in iodine. If rats are maintained on an iodine-deficient diet for about two years and groups of them are investigated with radioiodine and killed at intervals, it is possible to follow the development of their goitres (Axelrad & Leblond, 1955; Taylor & Poulson, 1956). Unfortunately the thyroid gland of the rat does not behave like that of man, and hyperplasia tends to remain uniform and not become focal. Nodules do appear, but are trabecular in pattern, and their iodine uptake, as demonstrated on autoradiographs, is similar to that of the rest of the gland.

Since the nodules which develop in human goitres appear to be related to increased secretion of TSH, it might be suggested that they arose as the result of groups of follicles becoming more sensitive to the TSH. To investigate this, excised thyroid glands were taken straight from operating-theatre to laboratory, and slices of nodular and non-nodular tissue were studied in a Warburg apparatus with varying levels of added TSH. Only on one occasion was there any difference shown in the responsiveness of the two kinds of tissue (Taylor, S. & Moffitt, B., communication to the Annual Meeting of the American Goiter Association, held in New York, May 28–30, 1957).

A further study is in progress to determine by chromatography what pattern of iodinated amino acids is produced in the functioning nodules removed at thyroidectomy. Pitt-Rivers, Hubble & Hoather (1957) have put forward evidence suggestive of a partial failure to convert MIT to DIT in some of these nodules.

It should be clear by now that many problems still remain to be answered as to why the diffuse hyperplasia of the thyroid at puberty should become nodular with the passage of time in those individuals who are denied an adequate intake of iodine in their youth. There is no problem, however, in preventing the formation of nodules in the thyroid gland for, if the daily intake of iodine is maintained at 100–200 μg./day, such changes do not occur. There are no recorded adverse effects in man from adding 50–100 μg. of iodine to the daily diet, preferably by iodizing salt, but although the Medical Research Council recommended this measure in Great Britain in 1944 and again in 1948 (Medical Research Council, 1944, 1948), it has never been implemented.

Nodular goitre is a preventable disease—surely it ought to be prevented.

ACKNOWLEDGEMENTS

I wish to thank the Medical Research Council, the British Empire Cancer Campaign and the Chilean Iodine Educational Bureau for assistance in carrying out work reported here.

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USE OF RADIOACTIVE IODINE IN THE DIAGNOSIS OF THYROID DISORDERS

A. W. G. GOOLDEN M.B. B.S. D.M.R.T.

Medical Research Council
Radiotherapeutic Research Unit
Hammersmith Hospital, London

1 Tests of uptake by the thyroid
  a Thyroid uptake during the early phase
  b Thyroid uptake in the intermediate phase
  c Thyroid uptake at 24 hours
  d Thyroid clearance rate
2 Urinary excretion tests
3 Organic radioiodine tests
4 Use of ^{132}I
5 Use of drugs and hormones in conjunction with ^{131}I tests
  a Thyrotrophic hormone
  b Thyroid hormone
  c Antithyroid drugs
6 Factors which influence iodine metabolism
7 Conclusions
References

The use of radioactive iodine (^{131}I) in the investigation of thyroid function is now well established, but confusion exists because so many different types of test have been described and because there is no general agreement on which type of test is most suitable for routine diagnostic use. In the diagnosis of thyrotoxicosis ^{131}I tests have to a large extent replaced other tests, such as the measurement of the basal metabolic rate, which measure different aspects of thyroid metabolism. In the diagnosis of hypothyroidism ^{131}I tests are less reliable. All ^{131}I tests depend fundamentally on the rate of accumulation of ^{131}I by the thyroid or on the rate of discharge of labelled hormone from the gland. The rate of accumulation of ^{131}I may be measured directly by in-vivo methods or indirectly by measuring the amount of ^{131}I excreted in the urine. The relative merits of the various methods are compared below and the use of the short-lived isotope of iodine, ^{132}I, is also described. Finally, certain factors which modify thyroid function or influence iodine metabolism are considered in relation to their effect on ^{131}I tests.

1. Tests of Uptake by the Thyroid

Tests which depend on the direct measurement of uptake of a dose of ^{131}I by the thyroid may be considered according to whether they are made in the early phase (within the first hour after the dose is given), the intermediate phase (1–6 hours), or at 24 hours. It is convenient to subdivide the tests in this way because the problems associated with the physical measurement of ^{131}I in the thyroid and with the kinetics of iodine metabolism differ in each of these phases. All in-vivo measurements of thyroid uptake are subject to considerable technical difficulties (Myant, Honour & Pochin, 1949). Single measurements of thyroid uptake at any selected time are particularly liable to geometrical errors, but by using the slope or some other parameter of the curve of uptake these errors may be considerably reduced. Another problem which applies particularly to early thyroid uptake tests is that of differentiating between the radioactivity in the thyroid and that in the extra-thyroidal tissues. Several methods of correction for extra-thyroidal radioactivity have been described (Myant, Corbett, Honour & Pochin, 1950; Berson, Yalow, Sorrentino & Roswit, 1952; Larsson, 1955; Goolden & Mallard, 1958a).

a. Thyroid Uptake during the Early Phase

For this type of test the dose of ^{131}I has to be given intravenously. There are several advantages in measuring thyroid uptake during the early phase. During this period the rate of change of thyroidal radioactivity is greatest and the rate of uptake is not obscured by the simultaneous loss of labelled hormone from the gland. Several workers have reported satisfactory results with thyroid uptake tests that are completed within half an hour of an intravenous dose (Clarke & Aujard, 1954; Farran, 1958). The curve of uptake for the first ten minutes has also been used as a diagnostic test (Larsson & Jonsson, 1955), and in one series was found to be considerably more reliable than the 24-hour uptake in the diagnosis of hyperthyroidism (Higgins, 1959).

b. Thyroid Uptake in the Intermediate Phase

For this type of test the dose of ^{131}I may be given by mouth. Correction for extra-thyroidal radioactivity is usually made, but is of much less importance than in the early phase. Thyroid uptake is commonly measured at two hours, but McConahey, Owen & Keating (1956) reported that a test of uptake at six hours compared favourably with other ^{131}I tests. Several workers have found that thyroid uptake at one hour is a reliable criterion for differentiating between euthyroid and hyperthyroid patients (Kriss, 1951; Morton, Ottoman & Peterson, 1951; Crispell, Parson & Sprinkle, 1953). Pochin (1950) has recommended the use of the neck: thigh ratio at two hours. This method, where the result is expressed as the ratio of the counting rate over the neck to the counting rate over the thigh, has the advantage that it is independent of the dose and does not require measurement of a standard. The test described by Foote & Maclagan (1951) is similar in principle.

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c. Thyroid Uptake at 24 Hours

Thyroid uptake at 24 hours has been widely used as a diagnostic test, and because of its simplicity and reliability has been recommended as the most suitable test for routine use (Werner, Hamilton, Leifer & Goodwin, 1950). It has also been used as a standard test for investigating the effects of drugs or hormones on thyroid function. More recently, various workers who have compared a number of different ^{131}I tests in the same group of patients have found the 24-hour uptake to be the least reliable of all the tests (Goodwin, Macgregor, Miller & Wayne, 1951; Storaasli, MacIntyre, Friedell & Weigle, 1955; Fryers, 1956; McConahey *et al.* 1956; Goldberg & Fitzsimons, 1958; Clarke, Fairley, King & Milne, 1958).

The average range of values for the 24-hour uptake for normal subjects has been reported to be between 10% and 40% (Werner, Quimby & Schmidt, 1949), whilst in hyperthyroidism it is usually greater than 50%. Patients who are difficult to assess clinically frequently have uptakes between 40% and 50%, and in this group the test is of little diagnostic help. The clear distinction between euthyroid and hyperthyroid patients in some series (Fields & Leroy, 1952; Freedberg, Chamovitz & Kurland, 1952) may have been due to the way in which the patients were selected.

The 24-hour uptake may be very misleading in thyrotoxic patients, in whom the rate of metabolism of iodine is greatly increased, since the peak uptake may occur within a few hours and by 24 hours an appreciable amount of ^{131}I may have left the gland as labelled hormone. The 24-hour or 48-hour uptake, however, may sometimes be useful in the diagnosis of hypothyroidism when tests of early thyroid uptake are of little value.

d. Thyroid Clearance Rate

The thyroid clearance rate, introduced by Keating, Wang, Luellen, Williams, Power & McConahey (1949) and Myant, Pochin & Goldie (1949), may be expressed as the volume of plasma cleared of ^{131}I per minute. This parameter, since it depends on the rate of uptake at a given plasma concentration, will not be influenced by extra-thyroidal factors such as the renal iodide clearance rate or the rate of diffusion of ^{131}I into the extravascular tissues. It has, moreover, certain practical advantages: the result is independent of the dose, and plasma activity, which is high at this stage, can be accurately measured.

On theoretical grounds the thyroid clearance rate has been regarded as giving the best estimate of the capacity of the thyroid to accumulate iodine (Myant, 1952). It has accordingly been used as a basis of comparison with other tests of thyroid uptake. However, it has been pointed out that the ^{131}I clearance rate is not necessarily the same as the net rate at which the stable iodide in the plasma is removed by the thyroid and converted to hormone (Ingbar, 1955; Larsson, 1955). The accumulation of iodine by the thyroid involves two separate processes. The first is concerned with the concentration or trapping of iodide at a higher level than that in the plasma, while the second involves the binding of iodine into an organic form. Iodide trapped by the thyroid is in reversible equilibrium with iodide in the plasma, so that unless conversion to organic iodine occurs very rapidly ^{131}I concentrated by the thyroid will have time to equilibrate with plasma ^{131}I. Since the concentration of ^{131}I in the plasma falls continuously until the distribution of the test dose is complete, this equilibration might lead to a loss of some ^{131}I from the thyroid and consequently to an apparent fall in the clearance rate. (For a detailed discussion of these points see Wollman (1954), Berson & Yalow (1955), Larsson (1955).)

Ingbar (1955) concluded that the ^{131}I clearance rate differed appreciably from the net rate of removal of stable iodide by the thyroid. His estimations, however, were based on assumptions of which some are not valid. Berson & Yalow (1955), on the other hand, showed that both the normal and hyperactive thyroid appeared to be capable of binding all the iodide extracted from the plasma before escape back to the plasma could occur. The limiting factor in the rate of accumulation of iodine by the thyroid was the rate of trapping rather than the rate of binding. The absence of any discharge of the accumulated thyroidal radioactivity after the administration of thiocyanate, an observation confirmed by Vanderlaan (1955), was further evidence of the rapidity of organic binding of iodine. Such a discharge can be readily demonstrated when organic binding is impaired, and it is not disputed that in these circumstances thyroid clearance of ^{131}I lacks significance.

Myant, Pochin & Goldie (1949) and Berson *et al.* (1952) found that the thyroid clearance rate, as estimated by ^{131}I, was fairly constant for several hours after the dose. Larsson (1955) found that thyroid clearance was constant in euthyroid subjects and relatively so in hyperthyroid subjects. When, however, the peak uptake occurred rapidly there was a tendency for the clearance rate to decrease, but this may well have been owing to the early release from the thyroid of ^{131}I in an organic form. Benua, Dobyns & Ninmer (1955), for instance, detected labelled tri-iodothyronine in the plasma within an hour of the administration of a dose of ^{131}I. The fact that the thyroid clearance rate of ^{131}I has been found to change little during the first few hours after the dose suggests that the rate of organic binding is very high and that transfer of trapped iodide from the thyroid to plasma is negligible. Hence, for practical purposes, the ^{131}I clearance rate may be regarded as a suitable basis for diagnostic tests. Although in one series—Goodwin *et al.* 1951—there was an appreciable overlap, most workers have confirmed that thyroid clearance is reliable for distinguishing between euthyroid and hyperthyroid patients (Berson *et al.* 1952; Owen, McConahey, Keating & Orvis, 1955; Paley, Sobel & Yalow, 1955; Goolden, 1958).

2. Urinary Excretion Tests

It will be apparent that in-vivo measurement of the amount of ^{131}I in the thyroid is technically difficult to carry out. The radioactivity in a sample of urine, on the other hand, can be accurately determined. For this reason urinary excretion tests, which are indirect tests of thyroid function, are sometimes preferred to in-vivo tests of thyroid uptake.

Iodide is removed from the plasma almost entirely by the thyroid and kidneys. Values for the renal clearance of iodide are in good agreement, with a mean value of about 35 ml./min. in normal subjects (Myant, Pochin & Goldie, 1949; McConahey, Keating & Power, 1951; Berson *et al.* 1952). Values for thyroid clearance in normal subjects also agree fairly well (Myant, Pochin & Goldie, 1949; Berson *et al.* 1952), the average value being about 17 ml./min. Since the thyroid and kidneys are the only organs which normally compete for iodide, the ultimate amount of ^{131}I accumulated by either organ will merely reflect this competition and will not necessarily be an accurate indication of thyroid or renal function.

It might be expected that impaired renal function, by increasing the plasma concentration of ^{131}I, would result in an

increase in the uptake of ^{131}I by the thyroid, and this may, in fact, occur (Skanse, 1949); more commonly, the accumulation of ^{131}I as judged by the 24- or 48-hour uptake is not increased. Perry & Hughes (1952) found that renal clearance of iodide was reduced in renal disease, as a result of which there was chronic retention of iodide and an increase in the plasma concentration of iodide. Although the thyroidal clearance of ^{131}I was reduced, this did not imply a reduction in thyroidal accumulation of stable iodide. Since both thyroid and renal clearance rates are decreased in renal disease, the ultimate accumulation of ^{131}I by the thyroid is not greatly altered although the cumulative curve of uptake is prolonged.

The simplest form of urinary excretion test consists of measuring the proportion of the dose excreted during a standard period, usually 24 or 48 hours. This type of test is not very discriminating and there is considerable overlap between the values in the different groups (Keating, Power, Berkson & Haines, 1947; Skanse, 1949).

Keating and his associates (Keating *et al.* 1947) investigated the urinary excretion of ^{131}I in people with normal and abnormal thyroid function. From an analysis based on the fractionated collection of urine they calculated the "extrarenal disposal rate" which represented the proportional rate of disappearance into sites other than the kidney. The extrarenal disposal rate, which reflected closely the accumulation rate when this was estimated directly from in-vivo measurements over the thyroid, was considered to be a satisfactory measure of the iodine-accumulating function of the thyroid, equivalent to the thyroid clearance rate (Keating *et al.* 1949; Luellen, Keating, Williams, Berkson, Power & McConahey, 1949). Clinical experience showed that the extrarenal disposal rate was a better index of thyroid function than the 24-hour thyroid uptake, the 48-hour urinary excretion, or the accumulation rate obtained from in-vivo measurements (Keating, Haines, Power & Williams, 1950).

Mason & Oliver (1949) and Arnott, Emery, Fraser & Hobson (1949) found that a more accurate estimate of thyroid function was obtained if the urine was collected over selected intervals. This observation forms the basis of the "*T*" index devised by Fraser, Hobson, Arnott & Emery (1953). This is an index based on the proportion of a test dose excreted during three periods (0–8, 8–24 and 24–48 hours), and is relatively independent of renal function. In practice there is very close agreement between the results given by the *T* index and the 8–24-hour excretion fraction (Goldberg & Fitzsimons, 1958). Hence the 8–24-hour excretion fraction alone may be used as a diagnostic test for hyperthyroidism, provided that accurate collection of urine is ensured; but collection in several fractions has the advantage that failure to make a complete collection is usually apparent from the pattern of excretion. Perhaps one of the main advantages of the *T* index is that it is a useful diagnostic test throughout the whole range of thyroid function, including hypothyroidism, thyroid uptake tests being of little value in this respect.

3. Organic Radioiodine Tests

^{131}I which has been accumulated by the thyroid is incorporated into thyroid hormone and reappears in the circulation bound to protein. Most of the protein-bound iodine (PBI) in plasma consists of thyroxine, but small amounts of tri-iodothyronine may also be present. The rate at which labelled hormone appears in the circulation depends on the turnover of iodine in the thyroid, but the level of $PB^{131}I$ in the plasma at a given time is a rather complex function which is influenced by the following factors: the proportion of the dose accumulated by the thyroid, the rate of secretion of hormonal iodine, the amount of organic iodine in the thyroidal and extrathyroidal pools, and the rates of degradation and faecal excretion of organic iodine. Despite this complexity, the level of $PB^{131}I$ in the plasma is a fairly sensitive index of hormone secretion, provided that the amount of organic iodine in the thyroid is relatively normal.

Various methods have been used to estimate the concentration of labelled hormone in the plasma. The organic iodine may be separated from the inorganic iodine by precipitation of the protein with a reagent such as trichloroacetic acid, or the thyroxine-like fraction may be separated by butanol extraction; or the inorganic iodine may be extracted by means of a suitable ion-exchange resin (Slade, 1956; Zieve, Vogel & Schultz, 1956).

It was suggested by Freedberg, Ureles & Hertz (1949) that $PB^{131}I$ estimations might be useful for diagnostic purposes, since at 24 hours the level of $PB^{131}I$ in the plasma differs considerably in euthyroid and hyperthyroid subjects. This difference is even more marked at 48 hours, by which time a plateau has usually been reached, and $PB^{131}I$ estimations are preferably carried out at this time. Clark, Moe & Adams (1949) introduced the conversion ratio, which is the percentage of the total ^{131}I in the plasma which is bound to protein. They found this an effective test for separating euthyroid from hyperthyroid patients, there being no overlap between the two groups. A slight disadvantage of the test is that renal insufficiency and congestive cardiac failure increase the level of inorganic ^{131}I in the plasma and thereby influence the conversion ratio. The standard method of expressing the results of $PB^{131}I$ estimations as a percentage of the administered dose per litre of plasma is free of this disadvantage.

Tests based on the accumulation of ^{131}I by the thyroid are more directly related to the production of hormone than are tests based on the release of organic ^{131}I from the gland (Riggs, 1952). In practice, however, $PB^{131}I$ tests appear to be at least as efficient for detecting hyperthyroidism, in patients who have not been previously treated, as tests which measure the iodine-concentrating capacity of the thyroid (Silver, Fieber & Yohalem, 1952; Paley *et al.* 1955; McConahey *et al.* 1956; Clarke *et al.* 1958), and in some series they have proved more reliable than any of the other ^{131}I tests with which they were compared (Goodwin *et al.* 1951; Clarke & Aujard, 1954; Wayne, 1954; Newburger, Silver, Yohalem & Feitelberg, 1955). They have not been found to be of any value in the diagnosis of hypothyroidism.

It is now generally recognized that $PB^{131}I$ tests are often misleading in patients who have been treated previously for thyroid disease. Ansell, Macgregor, Miller & Wayne (1953) first noted that patients who were euthyroid after treatment by surgery or ^{131}I often had high $PB^{131}I$ values, an observation subsequently confirmed by several other workers (Ingbar, Freinkel, Hoeprich & Athens, 1954; Horst, 1954; Lindeboom, Hoogendijk-van Dort & de Jong, 1955; Paley *et al.* 1955). Similarly, high $PB^{131}I$ values have been found in patients who were myxoedematous after thyroid surgery (Blom & Terpstra, 1953). The amount of organic iodine in the thyroid is often considerably reduced in patients who have been treated with ^{131}I (Berson & Yalow, 1954). Hence labelled hormone may appear rapidly in the plasma though the total daily output of hormone from the gland is within normal limits. $PB^{131}I$ tests

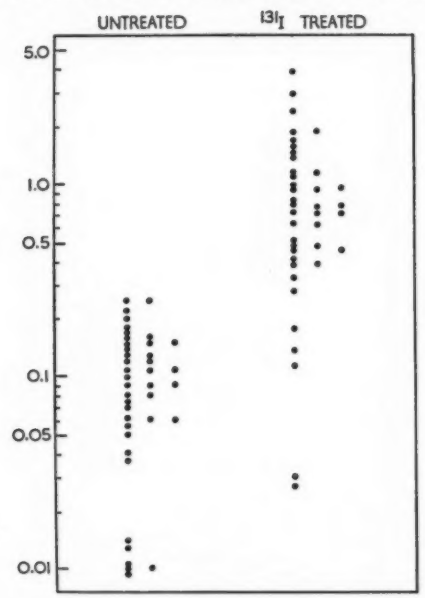
are, therefore, of little value in the diagnosis of residual or recurrent thyrotoxicosis, since abnormally high values are so frequent in patients who have been successfully treated for thyrotoxicosis by ^{131}I (fig. 1).

4. Use of ^{132}I

One of the principal advantages of ^{132}I, which has a half-life of 2.3 hours (Emery & Veall, 1954), is that the radiation dose is substantially less than that from ^{131}I. It should be used, therefore, in preference to ^{131}I when radioiodine tests are considered necessary in pregnancy or childhood, or for serial tests on the same person, in order to reduce to a minimum the radiation dose to the thyroid.

^{132}I is a daughter product of ^{132}Te, from which it may be separated by a simple distillation process (Cook, Eakins & Veall, 1956), or alternatively by elution from sodium tellurite adsorbed on a column of alumina (Stang, Tucker, Doering, Weiss, Greene & Banks, 1958). The availability of ^{132}I is therefore determined by the half-life of ^{132}Te, namely 77 hours. The ^{132}I after separation is contaminated with a small amount of ^{131}I, which has been found to vary from 0.07% to 2% (Goolden & Mallard, 1958b). The principal physical properties of ^{132}I and ^{131}I are compared in Table I. Owing to the higher energies of the β and γ emissions, the sensitivity of most counting systems will be greater for ^{132}I than ^{131}I by a factor of about 2. Although, for an equivalent number of μc in the gland, the radiation dose to the thyroid from ^{132}I is approximately one-thirtieth that of ^{131}I, the actual radiation dose to the thyroid for the same number of μc administered will be considerably less than one-thirtieth, since the amount of radioactivity concentrated by the thyroid is limited by the rapid decay of the isotope.

FIG. 1. Effect of Treatment on $PB^{131}I$ Values



Ordinates: $PB^{131}I$ (percentage of dose/litre of plasma)

$PB^{131}I$ values in 40 untreated euthyroid patients, and in 40 patients who, 3–4 months after treatment with ^{131}I for thyrotoxicosis, were assessed as euthyroid. The abnormally high range of values in the treated group is apparent.

TABLE I. Comparison of Principal Physical Properties of ^{131}I and ^{132}I

Property	^{131}I	^{132}I
Half-life	8.04–8.16 days	2.26 hours (Emery & Veall, 1954)
Average β-ray energy	0.197 MeV	0.45 MeV (Emery & Veall, 1954)
K factor for γ rays (r./h./mC at 1 cm.)	2.25	12.1 (Emery & Veall, 1954)
Total thyroid dose (30 g. spherical gland, 1 μC/g. initial concentration). Biological half-life, 120 days	122 rads	4.0 rads

^{132}I is especially convenient for serial tests in the same person, because the radioactivity from each test declines so rapidly that it does not interfere with the subsequent one. Studies, using ^{132}I, of the duration of effect of antithyroid drugs such as carbimazole or perchlorate, or the day-to-day changes in thyroid uptake as a result of the administration of thyroid-stimulating hormone (TSH) or thyroid hormone, have shown that it is particularly suited to this type of investigation (Goolden & Mallard, 1958b; Halnan & Pochin, 1958). The effect of TSH on thyroid function in normal subjects has been examined in some detail by Einhorn (1958). Observations on the thyroidal accumulation of radioiodine, which were made at relatively short intervals, were carried out with ^{132}I. Several workers have used ^{132}I to study variations in thyroid function in normal subjects (Hanbury, Heslin, Stang, Tucker & Rall, 1954; Fellinger, Höfer & Vetter, 1955; François, Goldberg, Goolden, McKinnell & Mallard, 1958); and Halnan (1958) has used it to study thyroid uptake during pregnancy.

Although ^{132}I has been available for several years, it has not so far been used to any extent for standard diagnostic work. The short half-life of the isotope restricts its use to early or intermediate tests of thyroid uptake, and it is best suited to some simplified test such as the neck:thigh ratio, which does not require any correction to be made for radioactive decay (Halnan & Pochin, 1958).

5. Use of Drugs and Hormones in Conjunction with ^{131}I Tests

a. Thyrotrophic Hormone

Querido & Stanbury (1950) showed that primary hypothyroidism could be differentiated from secondary hypothyroidism by observing the response of the thyroid gland to TSH. In patients with hypopituitarism, thyroid function, as judged by the uptake of ^{131}I, was increased after repeated

injections of TSH, whereas no appreciable change occurred in patients with primary thyroid disease. Other workers (Perloff, Levy & Despopoulos, 1951; Schneeberg, Perloff & Levy, 1954) using similar techniques have confirmed these observations, and have claimed that the response of the thyroid to stimulation by TSH is a useful means of assessing mild degrees of hypothyroidism. Jefferies and his associates (Jefferies, Levy, Palmer, Storaasli & Kelly, 1953) described a simplified method in which the thyroid uptake of ^{131}I at three hours after the dose was measured before and after a single injection of TSH.

Fletcher & Besford (1958), using the 24-hour uptake of ^{131}I as an index of thyroid function, have defined the normal response to TSH in various conditions. After a single injection of 10 United States Pharmacopeia (USP) units, the uptake in normal persons always tended to be within the range 50–60%, irrespective of the initial value. In hypopituitarism the uptake increased by a factor of at least 1.5 after an injection of TSH, whereas there was no response in primary myxoedema. It seems that considerably smaller doses of TSH than have been used previously may be equally effective for TSH tests. In normal subjects the maximum effect on thyroid uptake of ^{131}I is obtained with doses of 0.025 USP units of TSH/kg. of body-weight (Einhorn & Larsson, 1959).

The investigation of thyroid disorders in this way has revealed a condition of "low thyroid reserve" in which a remnant of thyroid tissue, under maximum stimulation by endogenous TSH, is capable of maintaining a normal output of hormone, but fails to respond to further stimulation by exogenous TSH (Jefferies, Kelly, Levy, Cooper & Prouty, 1956). A similar abnormality has been noted in a major proportion of patients who are euthyroid after treatment with ^{131}I for thyrotoxicosis (Martin & Stanbury, 1955), and in patients with Hashimoto's disease (Skillern, Crile, McCullagh, Hazard, Lewis & Brown, 1956).

b. Thyroid Hormone

It has been shown that thyroid uptake of ^{131}I is inhibited in normal subjects by the administration of desiccated thyroid (Stanley & Astwood, 1949; Greer, 1951), thyroxine (Morgans, Oldham & Trotter, 1952), or tri-iodothyronine (Starr & Liebhold-Schueck, 1953). In the case of tri-iodothyronine the dose-response relationship has been studied (McConahey & Owen, 1956). Doses greater than 25 μg./day consistently suppress thyroid uptake after about a week. As the dose is increased to 140 μg./day inhibition occurs more rapidly. The rate of secretion of hormone is also inhibited in normal subjects given either thyroxine or tri-iodothyronine (75–100 μg./day) (Johnson, Solomon & Greer, 1959). In hyperthyroidism, however, thyroid function is not suppressed by the administration of any thyroid hormone. Massive doses (2 mg./day) of tri-iodothyronine affect neither thyroid uptake of ^{131}I (Werner & Hamilton, 1953) nor the rate of secretion of hormone (Johnson *et al.* 1959). Thus it is possible to distinguish euthyroid from hyperthyroid patients by their response to desiccated thyroid (Greer & Smith, 1954) or tri-iodothyronine (Werner & Spooner, 1955; Perlmutter & Slater, 1955). This suppression test is useful when a high uptake of ^{131}I is found in patients with non-toxic goitre. In these circumstances thyroid uptake is usually, though not invariably, suppressed after the administration of tri-iodothyronine (Dresner & Schneeberg, 1958).

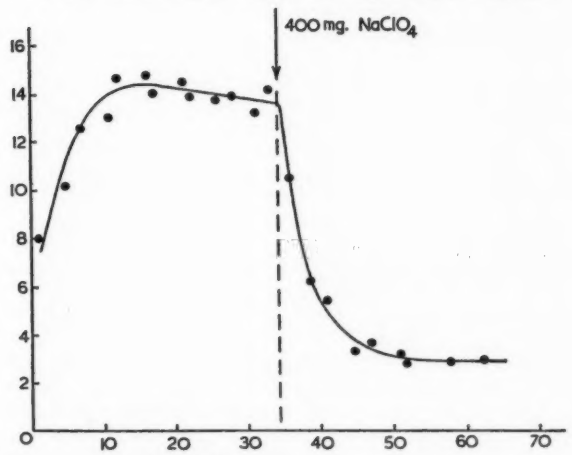
Werner (1956) has studied the effect of tri-iodothyronine on thyroid uptake in patients in remission from hyperthyroidism after treatment by surgery or ^{131}I. In most of the patients treated by ^{131}I, tri-iodothyronine failed to suppress thyroid uptake, and this abnormality often persisted for many years. Lack of suppression also occurred in some patients treated by surgery, but more often a normal response was obtained. Morgans *et al.* (1952) also studied patients in remission from thyrotoxicosis after treatment by surgery, and found that thyroid uptake was suppressed, as in normal subjects, after the administration of thyroxine.

c. Antithyroid Drugs

Vanderlaan & Vanderlaan (1947) showed that in the rat the thyroid could still concentrate iodide even when binding of iodine was prevented with propylthiouracil. Iodide, thus accumulated in the thyroid, could be discharged by thiocyanate, which also inhibited the iodide-concentrating mechanism. Stanley & Astwood (1948) made similar observations in man. The iodide trap is to some extent an artificial concept which can be demonstrated only when iodine binding is inhibited with a thiouracil-like drug. Under these conditions ^{131}I is accumulated in the thyroid, rapidly reaching a maximum value, and is then retained as iodide in the gland, whence it can be discharged by thiocyanate or perchlorate (see fig. 2). Stanley & Astwood (1948) found that the iodide-concentrating mechanism was greatly enhanced in thyrotoxicosis and suggested that a measure of this function might provide an index of thyroid activity. Childs, Keating, Rall, Williams & Power (1950), using large doses of iodide to inhibit iodine binding, derived a quantitative value for the magnitude of the iodide trap from the ratio:

$$\frac{\text{percentage of dose of } ^{131}I \text{ in thyroid}}{\text{percentage of dose of } ^{131}I/\text{l. plasma}}$$

FIG. 2. The Iodide Trap in a Thyrotoxic Patient



The thyroid uptake of ^{131}I in a thyrotoxic patient when organic binding of iodine has been inhibited. Carbimazole (60 mg.) was given one hour before the test dose of ^{131}I. Sodium perchlorate (400 mg. intravenously) caused a prompt discharge of inorganic iodide from the gland. The value for the thyroid iodide space in this patient was 2.8 litres.

The value thus obtained, in litres, they designated the thyroid iodide space. The mean value in thyrotoxic patients was 9.5 l., while in euthyroid patients it appeared to be about 1 l. or less. Newsholme (1952), using methylthiouracil or sodium iodide to prevent iodine binding, found that the neck:thigh ratio was a useful index of the iodide-concentrating function of the thyroid. There was a good correlation between this ratio and the thyroid iodide space. This type of test does not seem to have been generally adopted but might be useful in patients, for instance, who are already having treatment with an antithyroid drug.

Morgans & Trotter (1957) found that, in patients with Hashimoto's disease, perchlorate given an hour after the tracer dose of ^{131}I caused an appreciable loss of radioactivity from the thyroid, owing to discharge of inorganic iodide. No such loss occurred in normal subjects or in patients with simple goitre. It was concluded that there was a defect in the binding of iodine in this condition. An alternative explanation might be that the thyroid iodide space was increased without there being a proportionate increase in the binding rate. It is of interest that Roche, de Venanzi, Vera, Coll, Spinetti-Berti, Mendez-Martinez, Gerardi & Forero (1957) found that perchlorate produced a similar loss of inorganic iodide from the thyroid in patients with endemic goitre.

6. Factors which Influence Iodine Metabolism

As early as 1939 Hamilton & Soley noted that some patients with non-toxic nodular goitres had an increased uptake of ^{131}I, and a later report by Hamilton, Soley, Reilly & Eichorn (1943) described two hypothyroid children with large goitres and high uptakes. Since then there have been a number of reports of a high uptake of ^{131}I in patients with goitrous cretinism, and it has been found that the avidity of the thyroid for iodine is increased in a number of conditions other than thyrotoxicosis. If ^{131}I tests are to be interpreted correctly, various factors which may influence the kinetics of iodine metabolism must be recognized. One of the most important of these factors is iodine deficiency.

Most cases of endemic goitre are now generally thought to be due to iodine deficiency. Stanbury, Brownell, Riggs, Perinetti, Del Castillo & Itoiz (1952) and Stanbury, Brownell, Riggs, Perinetti, Itoiz & Del Castillo (1954) made a study of iodine metabolism in the inhabitants of Mendoza, an endemic goitre area in western Argentina. The uptake of ^{131}I in these people was very high and showed an inverse correlation with the amount of stable iodide excreted in the urine. Most of the iodide derived from degradation of hormone was being re-utilized. The thyroidal store of organic iodine was low and the rate of turnover of iodine in the thyroid was increased, but the level of $PB^{127}I$ in the plasma was within normal limits. Similar studies with ^{131}I were carried out in an endemic goitre area in Venezuela (Roche, de Venanzi, Spinetti-Berti, Gerardi, Mendez-Martinez & Forero, 1956; Roche *et al.* 1957). Thus the development of a hyperplastic gland, or goitre, represents the compensatory mechanism whereby the thyroid gland of a person subsisting on an iodine-deficient diet is able to conserve the limited amount of iodine available. The response of the thyroid to iodine deficiency is probably initiated and maintained by thyrotrophic hormone, and thyroid function can be suppressed by the administration of thyroid hormone, the hyperplastic gland behaving in this respect in the same way as the normal gland. These reports from Mendoza and Venezuela describe the response of the thyroid gland to severe iodine deficiency. Lack of iodine in the diet in non-endemic areas is probably uncommon, but was thought to be the cause of a high uptake of ^{131}I in a group of patients on a rice diet (Bishopric, Garrett & Nicholson, 1955). The increased uptake sometimes found in patients with cirrhosis of the liver may also be dietary in origin (Shipley & Chudzik, 1957).

Iodine deficiency may also occur from failure to utilize iodine properly, as for example in patients taking drugs which have an antithyroid effect, which, if taken for prolonged periods, frequently give rise to goitre and hypothyroidism. It has been shown that most of these substances, which include amphenone (Selenkow, Rivera & Thorn, 1957), *p*-aminosalicylic acid (Macgregor & Somner, 1954), resorcinol (Bull & Fraser, 1950), and phenylbutazone (Morgans & Trotter, 1955), inhibit organic binding of iodine. Other substances, such as salts of cobalt (Kriss, Carnes & Gross, 1955) and the sulphonyl urea derivatives, carbutamide and tolbutamide (Brown & Solomon, 1956), appear to interfere with the biosynthesis of hormone, but their exact mode of action has not been defined.

The thyroid reacts to iodine deficiency in the first place by an increase in the thyroid clearance rate, but there is no concomitant increase in the turnover of iodine in the thyroid, the $PB^{131}I$ level being within normal limits. This apparent discrepancy between iodine uptake and hormone secretion is sometimes seen in patients with non-toxic goitre; it is important to realize that in these circumstances any test which measures the accumulation of ^{131}I is likely to be misleading. It has been found that about 10–20% of patients with non-toxic goitre have a high uptake (Keating *et al.* 1950; Fraser *et al.* 1953), but thyrotoxicosis can usually be excluded in this group by a $PB^{131}I$ estimation (Wayne, 1954).

It is generally realized that ^{131}I tests may be very misleading in patients who have been recently treated with antithyroid drugs, thyroid hormone or compounds containing iodine. Some of the effects of antithyroid drugs and thyroid hormone have already been mentioned. Thyroid function may be depressed for several weeks in patients who have been taking thyroid extract or thyroxine, but recovery occurs more rapidly after stopping tri-iodothyronine, returning to normal usually within about a week. These iodine compounds contain enormous amounts of iodine compared with the average daily intake, which is about 150 μg./day (Riggs, 1952). They affect ^{131}I tests in the first place by flooding the iodide space with large amounts of stable iodide and so reducing the specific activity. In these circumstances the accumulation of ^{131}I by the thyroid is not an indication of the amount of stable iodide being transferred from plasma to thyroid. The pharmacological effects of iodide on thyroid function are rather complex and by no means fully understood. Moreover, the thyrotoxic gland does not necessarily react to iodide in the same way as the normal gland. For instance, doses of about 1 mg. of potassium iodide inhibit organic binding in thyrotoxicosis, though the effect may not be permanent (Childs *et al.* 1950), but do not influence significantly ^{131}I uptake in normal subjects. Similarly, iodide reduces the rate of secretion of hormone in thyrotoxicosis (Ansell & Miller, 1952) but has no effect in euthyroid subjects (Greer & DeGroot, 1956). Iodide, which is contained in a great variety of drugs and preparations, may sometimes have an antithyroid effect in euthyroid persons, giving rise to goitre and hypothyroidism. The organic iodine compounds used as *x*-ray contrast media are another

source of iodine, some of them being retained in the body for months or years, during which time they continue to liberate iodide.

It can be seen that ^{131}I tests are of limited value when there are considerable variations from the normal in the amount of ^{127}I in the iodide pool. Thus the uptake of ^{131}I merely indicates the fraction of the iodide pool accumulated by the thyroid in a certain time. Since the total quantity of ^{127}I in the iodide pool is not known, ^{131}I tests do not give any information on the amount of ^{127}I entering the thyroid. It might be expected that the uptake of ^{127}I would provide a better index of thyroid activity than the uptake of ^{131}I. Burrows & Ross (1953) compared thyroidal uptake of ^{131}I and ^{127}I and found that the latter was more closely related to the $PB^{127}I$ level.

7. Conclusions

The problems involved in the interpretation of the results of ^{131}I tests have been discussed in some detail: in practice, difficulties arise in only a small proportion of patients. ^{131}I tests are on the whole very satisfactory for detecting hyperthyroidism and are particularly useful in certain hyperthyroid patients, notably those with thyrocardiac disease, who may have very few of the signs or symptoms of thyrotoxicosis.

It is unreasonable to expect that any ^{131}I test will distinguish every patient as being either euthyroid or hyperthyroid. Clinical experience shows that there is no sharp transition between euthyroid and hyperthyroid states. In doubtful cases the diagnosis is usually resolved by observing whether there is any response to treatment with an antithyroid drug.

In the diagnosis of hyperthyroidism, early or intermediate tests of thyroid uptake and $PB^{131}I$ tests appear to be equally reliable. An early uptake test, although requiring an intravenous injection, is very convenient for the patient and is completed within half an hour. An excretion test such as the *T* index, though less convenient for the patient, compares favourably with other tests provided that accurate collection of urine is ensured, but results are sometimes invalidated because of incomplete collection. In the diagnosis of hypothyroidism an excretion test is usually more reliable than other ^{131}I tests.

As a general rule, an uptake test used in conjunction with a $PB^{131}I$ test is the most satisfactory method of assessing thyroid function. The combination of a high uptake and a high $PB^{131}I$ value is nearly always due to thyrotoxicosis. A suppression test with tri-iodothyronine may be used if further confirmation is needed. Patients who have been treated with ^{131}I are less easily assessed, since the $PB^{131}I$ test is of no diagnostic value, and a high uptake may occasionally persist despite a return to the euthyroid state.

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SPORADIC GOITROUS CRETINISM

E. M. McGIRR B.Sc. M.D. M.R.C.P. F.R.F.P.S.G.

Senior Lecturer in Medicine
University of Glasgow

Department of Medicine
Royal Infirmary, Glasgow

1 Historical background
2 Survey of various biochemical anomalies described in sporadic goitrous cretinism
  a Failure to produce organic iodine compounds despite a facility to trap inorganic iodide
  b Failure to conserve iodine of the iodotyrosines because of lack of the enzyme dehalogenase
  c Failure to couple iodotyrosines to produce iodothyronines
  d Production and secretion of an abnormal iodinated thyroid protein or polypeptide
3 Conclusions
References

1. Historical Background

"... nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of nature, by the careful investigation of cases of rarer forms of disease."

William Harvey (1657)

Goitrous cretinism immediately suggests the classical type of endemic cretinism with an enlarged thyroid gland, which is believed to occur only in highly endemic goitrous areas. Such cases are due in large part to iodine deficiency, but intermarriage probably also plays an important role in their causation. In contrast, the common type of sporadic cretinism is most probably due to a developmental anomaly, and thyroid tissue is absent or rudimentary. Sporadic goitrous cretinism comprises those cases of cretinism with enlarged thyroid glands which occur outwith highly endemic goitrous areas, for example in countries such as the United Kingdom. They are uncommon even in the areas where goitre is most common, and, as far as I am aware, there is no evidence that they are due to primary iodine deficiency. It is in practice impossible to separate these cases into so-called endemic and sporadic categories and it serves no useful purpose to attempt to do so. Some of these cases are due to the action in utero or after birth of more or less well-recognized goitrogens or to Hashimoto's thyroiditis. Others cannot, however, be explained in these ways. They were recognized by nineteenth-century clinicians, but their occurrence aroused little interest until recently.

As far as can be ascertained the only instance in England of anything approaching to an endemic of cretinism occurred in the village of Chiselborough in Somerset in the first half of last century (Norris, 1848). Among the population of 540, there were 4 complete cretins, 17 semi-idiots and 5 deaf-mutes. The majority of the community were dull-witted and were goitrous. In the fashion of those days the condition was attributed to a combination of circumstances such as the effects of a water supply of inferior quality, impure air and intermarriage. In the words of Norris (1848):

> . . . circumstances concurring thus to render the parish so uninviting to strangers, the poor inhabitants were under the necessity of "putting up" with each other, and thus, perhaps, unwittingly completing the unfortunate combination of circumstances which may have produced such miserable results.

By 1896 only one solitary cretin survived (Parker, 1896). The factors credited with the eradication of the condition were improved sanitary measures, better food, better education and greater contact with the outside world as the result of the opening up of secluded valleys. Parker thought that it had occurred "... in the worst breeding grounds of goitre (namely, in valleys where the right conditions for goitre happen to exist) and which are sufficiently secluded and benighted to induce frequent intermarriage among goitrous families."

Osler (1897) also recognized the familial tendency to goitrous cretinism and he commented upon the occurrence of consanguinity among the parents of the cretins. In a survey of cretinism in the United States of America he found a low but definite incidence of goitre. An enlarged thyroid gland was present in 7 out of 60 sporadic cretins. In one family of 5 children 3 were sporadic goitrous cretins.

These early observations can be seen in retrospect to suggest a genetic basis for sporadic goitrous cretinism. They are all the more noteworthy because nothing more was added to our knowledge of goitrous cretinism, nor indeed was much attention devoted to it until 1943 when Hamilton, Soley, Reilly & Eichorn first used radioactive iodine (^{131}I) in the investigation of hypothyroid children. Since 1943 cases of sporadic goitrous cretinism have aroused increasing interest. Evidence from biochemical and genetic studies has been gradually accumulated to support the belief that this type of cretinism is due to intrinsic defects in the synthesis of the thyroid hormone and that these defects are genetically determined (Lelong, Joseph, Canlorbe, Job & Plainfosse, 1956; Stanbury & McGirr, 1957; Hutchison, 1958).

2. Survey of Various Biochemical Anomalies Described in Sporadic Goitrous Cretinism

Consideration of the sequence of reactions by which the thyroid hormones are synthesized reveals that there are ample opportunities for defects in synthesis to occur. Several defects have already been more or less well defined. They may be classified as follows.

a. Failure to Produce Organic Iodine Compounds despite a Facility to Trap Inorganic Iodide

This defect is readily unmasked by an oral dose of thiocyanate or perchlorate. These anions discharge any ^{131}I accumulated by the thyroid gland that has persisted as iodide and has not been incorporated into tyrosines and thyronines. The presumption is that there is a deficiency in the peroxidase enzyme system (Stanbury & McGirr, 1957). Alternatively, but probably much less likely, this defect might be due to a circulating metabolite with a thiourea-like action produced as the result of an enzyme block in another tissue (Trotter, 1959).

A defect in the conversion of iodide to organic iodine has been found both in goitrous cretins and in euthyroid patients with non-toxic nodular goitres. It was first described in goitrous cretinism by Stanbury & Hedge (1950) who studied three of four goitrous cretins in a family of seven. Other cases

have been reported by Stanbury (1951); Jackson (1954); Lelong *et al.* (1956); Schultz, Flink, Kennedy & Zieve (1957); Buchanan & Crooks (1959); and Gardner, Hayles, Woolner & Owen (1959). Cases of sporadic goitre with a similar defect have been recorded by Clayton, Smith & Leiser (1958), Morgans & Trotter (1958), and McGirr, Hutchison & Clement (1959b).

A notable additional finding in the euthyroid patients, reported by Morgans & Trotter (1958) and McGirr *et al.* (1959b), was deafness or deaf-mutism. I have recently seen two goitrous and cretinous siblings with a peroxidase defect. They were also deaf. It is tempting to suggest that the cretins in Somerset described by Norris (1848), among whom there were deaf-mutes, had a similar biochemical defect. The association of goitre and deaf-mutism has been recognized for a very long time (Bircher, 1883) but the relationship between them remains unexplained.[1] Deaf-mutism is well known often to be hereditary. The familial incidence of cases of goitrous cretinism and of sporadic goitre with a defect in conversion of iodide to organic iodine suggests that this anomaly has a genetic basis. Published evidence is not yet sufficient to justify any definite conclusion as to whether a single genetic defect is the cause of two embryologically unrelated lesions (Trotter, 1959) or whether two closely linked genes are involved (McGirr *et al.* 1959b).

Chromatographic analysis of the ^{131}I compounds present in thyroid tissue of goitrous cretins with a peroxidase defect virtually fails to reveal any organic ^{131}I compounds (Stanbury, Ohela & Pitt-Rivers, 1955). In one of my patients, for example, more than 99.5% of the ^{131}I in the thyroid gland was present as iodide 18 hours after an oral dose of 100 μc ^{131}I. In the euthyroid goitrous patients with a peroxidase defect whom we studied in Glasgow (McGirr *et al.* 1959b), there was presumptive clinical evidence that enough hormone was produced to meet their metabolic needs. In these patients chromatographic analysis of thyroid tissue revealed the presence of iodotyrosines and iodothyronines, and supported the clinical deduction that the biochemical defect was not complete.

b. Failure to Conserve Iodine of the Iodotyrosines because of Lack of the Enzyme Dehalogenase

Monoiodotyrosine (MIT) and di-iodotyrosine (DIT) do not normally appear in the blood when they are released from storage in the colloid, because they are de-iodinated by the enzyme dehalogenase (Roche, Michel, Michel & Lissitzky, 1952; Roche, Michel, Michel, Gorbman & Lissitzky, 1953). Their iodine is presumably conserved and is re-utilized in the production of hormone. Credit for first demonstrating impaired dehalogenase activity in goitrous cretins must be given to Stanbury and his colleagues working in Leiden (Stanbury, Kassenaar, Meijer & Terpstra, 1955; Querido, Stanbury, Kassenaar & Meijer, 1956; Stanbury, Kassenaar, Meijer & Terpstra, 1956; Stanbury, Meijer & Kassenaar, 1956).

The most interesting group of cases with this defect is the tinker family-group which Dr James H. Hutchison and I have studied in Glasgow (McGirr & Hutchison, 1953; Hutchison & McGirr, 1954; Hutchison & McGirr, 1956; McGirr & Hutchison, 1956; McGirr, Hutchison & Clement, 1956; McGirr, Hutchison & Clement, 1959a). The pedigree of the main family-group is presented in fig. 1. It shows that 13 goitrous cretins appeared among 42 persons in five sets of siblings. It provides strong evidence for the belief that the dehalogenase defect which was demonstrated in these patients was dependent on the activity of a single recessive autosomal gene. In addition to the affected members of the main family-group, we also had the opportunity to study two isolated tinker families in which there were five goitrous cretins.

Evidence for a dehalogenase defect is built up by one or more of three different methods:

i. The identification by chromatography of iodotyrosines in the serum, or of free or conjugated iodotyrosines in the urine after a tracer dose of ^{131}I. A dose of 100 μc ^{131}I has rarely proved sufficient in our experience to give measurable amounts of iodotyrosines in the serum. The amount of radioactivity that is necessary obviously limits the usefulness of this method.

ii. The chromatographic analysis of the ^{131}I urinary excretion products after an intravenous or oral dose of D^{131}IT (Stanbury, Meijer & Kassenaar, 1956) or M^{131}IT (McGirr *et al.* 1959a). This is an attractive test because it requires the use of only small amounts of radioactivity, such as 5 μc. We have found that after an oral dose of M^{131}IT, euthyroid subjects used as controls, whether non-goitrous or goitrous, excrete less than 6% of it as free or conjugated M^{131}IT in the period 0–6 hours, whereas four of our tinker cretins and an 11-year-old euthyroid girl with a goitre excreted more than 28% (McGirr, Clement, Currie & Kennedy, 1959; McGirr *et al.* 1959a).

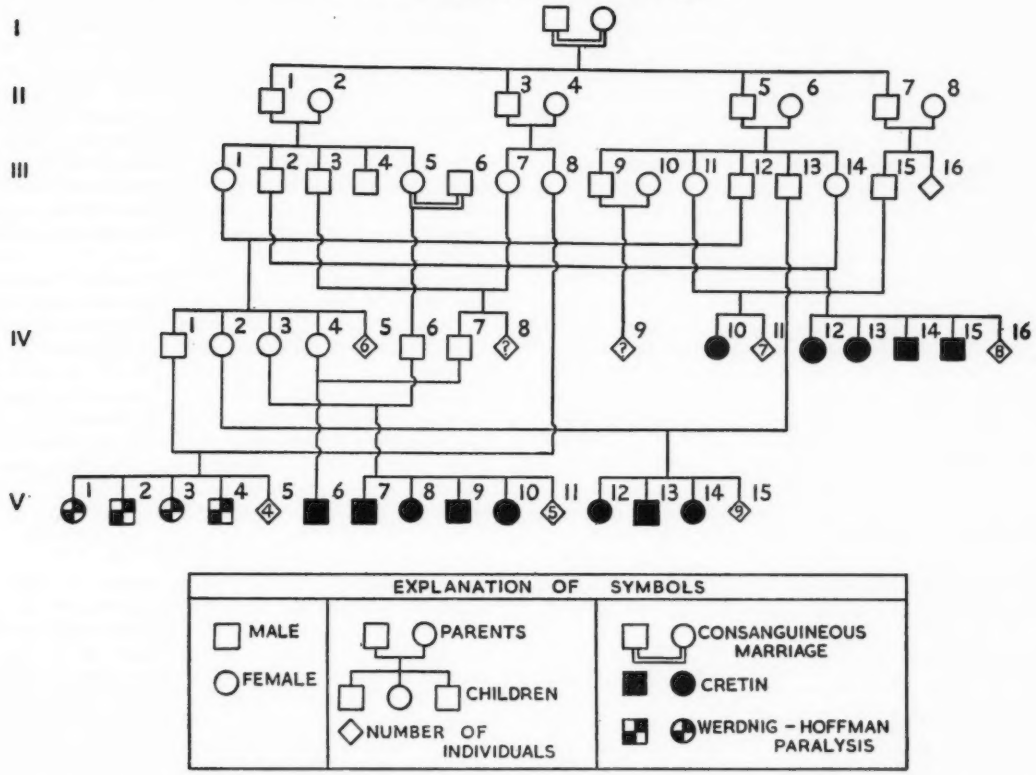
iii. The demonstration by in-vitro studies that fresh thyroid tissue fails to de-iodinate D^{131}IT (Querido *et al.* 1956) or M^{131}IT (McGirr *et al.* 1959a) under standard conditions. This test gives the ultimate proof of the absence of dehalogenase activity. As far as I am aware this has been shown on only three occasions, namely in a goitrous cretin in Leiden (Querido *et al.* 1956), in one of the tinker cretins in Glasgow (McGirr *et al.* 1959a) and in the 11-year-old girl referred to above (McGirr *et al.* 1959).

Our experience has been that thyroid tissue is rarely available to carry out an in-vitro test, and that the M^{131}IT excretion test has to be used as a practicable second-best test. This test may be used not only to reveal evidence of a dehalogenase defect but also on occasion to establish a common heredity among affected patients. For example, of the four tinker cretins so investigated in Glasgow, two belonged to the main tinker family and two were of uncertain ancestry. The M^{131}IT excretion test indicated that their enzyme defect was identical and supported our belief that the two isolated cases had a common ancestry with the main group (McGirr *et al.* 1959a). The M^{131}IT excretion test may at times unmask a minor biochemical abnormality in apparently normal subjects. A similar but less marked impairment of the de-iodination of M^{131}IT was found in four relatives of the tinker patients and in the mother of the 11-year-old girl. These relatives were euthyroid, non-goitrous and normal to outward appearances. Stanbury, Meijer & Kassenaar (1956), using D^{131}IT, also found evidence of a minor biochemical defect in four of five relatives of one of their goitrous cretins who had a dehalogenase defect; these relatives were euthyroid but were goitrous.

In the light of the results of the M^{131}IT and D^{131}IT excretion tests it appears to be a reasonable conclusion that the patients with frank goitre and hypothyroidism were homozygous, while the relatives who showed only minor biochemical

[1] See also Trotter, p. 92 of this number of the Bulletin, for a discussion of this problem.—Ed.

FIG. 1. Pedigree of Family-Group of Tinkers, Showing 13 Cases of Goitrous Cretinism and 4 Cases of Werdnig-Hoffman Paralysis



abnormalities were heterozygous carriers of the gene, which was in fact incompletely recessive (Stanbury & McGirr, 1957; McGirr *et al.* 1959a).

In general, the results reported with $D^{131}IT$ and $M^{131}IT$ excretion tests have been comparable, but occasionally inconsistencies occur. On the one hand, there is evidence that inability to de-iodinate iodotyrosines, in particular DIT, may sometimes be merely a secondary effect of hypothyroidism (Stanbury & Litvak, 1957; Mosier, Blizzard & Wilkins, 1958; Trotter, 1959) and that it is corrected when the patients are rendered euthyroid by thyroid replacement therapy (Mosier *et al.* 1958). On the other hand, we have found that the increased urinary excretion of $M^{131}IT$ in our tinker cretins was independent of their clinical status.

There appears to be little doubt that loss of the iodotyrosines is the outstanding biochemical effect of diminished or absent dehalogenase activity. This loss, directly or indirectly by virtue of loss of iodine, may account for the clinical state of the patients, but it is also possible that an additional defect is present, such as difficulty in coupling iodotyrosines to form iodothyronines (Gardner *et al.* 1959). There should be clear evidence of diminished activity of each of the enzymes inculpated if multiple defects are postulated, for multiple errors imply that more than one gene is defective. It is contrary to the concepts of modern genetics that one gene could be responsible for two enzymes.

c. Failure to Couple Iodotyrosines to Produce Iodothyronines

This defect was first postulated by Stanbury, Ohela & Pitt-Rivers (1955) in two goitrous sisters who showed the residual stigmata of cretinism. Thyroxine and tri-iodothyronine were present in the serum in normal amounts, but chromatographic analysis of the ^{131}I compounds in the thyroid gland of one of them showed that the iodine was present almost entirely as MIT and DIT; exhaustive chemical analyses revealed only a trace of thyroxine. These authors suggested that a small amount of iodinated thyronines was produced non-enzymically. Their findings were compatible with a primary failure in enzymic coupling. As convincing proof of the existence of a coupling enzyme is still lacking, it is not surprising that they could offer no direct proof of its failure.

Lelong *et al.* (1956), Werner, Block, Mandl & Kassenaar (1957) and Mosier *et al.* (1958) gave a similar interpretation to their findings in other cases of goitrous cretinism. Elsewhere I have described my findings in a ten-year-old euthyroid boy with a large congenital goitre (McGirr, 1960). Failure of potassium thiocyanate to discharge any ^{131}I from his thyroid showed that the initial steps in the utilization of iodide were normal. An oral dose of $M^{131}IT$ was de-iodinated normally, indicating normal dehalogenase activity in the tissues of the body, and presumably also in the thyroid. Chromatographic analysis of the thyroid gland after 100 μc ^{131}I showed radioactivity in zones corresponding to MIT and DIT, but no

thyroxine was detected. This boy's euthyroid state indicated that enough thyroid hormone was produced for his growth and metabolic needs. His thyroid was apparently able to produce and secrete an adequate amount of hormone but did not produce enough to store any appreciable amount of it. The findings were tentatively interpreted as being compatible with a coupling defect, which was apparently incomplete.

In some of the cases in which a coupling defect has been postulated (Werner *et al.* 1957; Mosier *et al.* 1958; Gardner *et al.* 1959), iodotyrosines were detected in the blood or urine after a dose of ^{131}I and there was evidence of impaired peripheral de-iodination of D^{131}IT. The presence of iodotyrosines in the blood or urine of patients with a so-called coupling defect has not been satisfactorily explained. It may be that excessive accumulation in their thyroids of MIT and DIT in conjunction with their hypothyroid state overwhelms a fundamentally normal dehalogenase system, which is temporarily at a disadvantage. Alternatively there may be some link between the enzymic coupling of the iodotyrosines and their enzymic de-iodination. Claims for the second alternative should be examined most critically because genetically it seems unlikely.

d. Production and Secretion of an Abnormal Iodinated Thyroid Protein or Polypeptide

Patients with this defect have an unusual iodinated serum compound which is precipitable with the proteins, but, unlike thyroxine, is insoluble in butanol. They are readily distinguished from others with biochemical evidence of dyshormonogenesis by the discrepancy between the amounts of their serum ^{131}I which are protein-precipitable and which are butanol-soluble after a tracer dose of ^{131}I (Di George & Paschkis, 1957; Stanbury & McGirr, 1957; De Groot, Postel, Litvak & Stanbury, 1958; McGirr, Hutchison, Clement, Currie & Kennedy, 1960). Theoretically they may be confused with patients with thyroid carcinoma (Robbins, Rall & Rawson, 1955; Tata, Rall & Rawson, 1956) and Hashimoto's thyroiditis (Owen & McConahey, 1956) who may have a similar unusual iodinated compound in their serum, but in practice such confusion is unlikely.

De Groot *et al.* (1958) investigated in some detail the properties of the compound in their patient, who was a 27-year-old woman. They found that it was non-dialysable and was sedimented with proteins in the ultracentrifuge. It moved with the serum albumin fraction on paper electrophoresis, but was apparently not part of, nor necessarily associated with, serum albumin, since it did not precipitate in the presence of albumin antiserum. Enzymic hydrolysis, particularly with chymotrypsin, liberated MIT, DIT and thyroxine. They suggested that the defect in their patient was the formation of an abnormal thyroprotein in the gland, or the abnormal fragmentation and release of normal thyroglobulin.

Between 1955 and 1959 we have seen six goitrous patients, five of whom were or had been at some time hypothyroid, with this defect. In the chromatographic analysis of the ^{131}I compounds in the thyroid gland of a 19-year-old euthyroid girl after a dose of 1 mc ^{131}I, we found an unusual iodinated compound. It was resistant to enzymic digestion, remaining insoluble in butanol, but it was degraded by alkaline hydrolysis, which revealed the only thyroxine present in the gland, as well as MIT and DIT (McGirr, 1960; McGirr *et al.* 1960). There were two unusual findings in the electrophoretograms[2] of the thyroid proteins (Watson, Yeung & McGirr, 1960), namely a broad band of radioactivity extending from the usual position of thyroglobulin (there was negligible stainable thyroglobulin) to a little in advance of albumin, and a distinct peak of radioactivity near the origin.

It seems probable that the unusual serum compound is a derivative of an unusual thyroid protein. The former, unlike the latter, may be susceptible to chymotrypsin because it has already been acted upon by thyroid protease. These patients may have insufficient circulating effective hormone either because thyroxine is not released from the thyroid in adequate amounts, or because thyroxine present in the abnormal serum compound is not susceptible to the appropriate de-iodinating enzyme.

Nothing is known about the relationship, if any, between our finding in thyroid tissue of a relatively large amount of radioactivity in and in front of the albumin zone of the electrophoretograms, and electrophoretic findings in serum described by De Groot *et al.* (1958). Likewise we do not know the significance of radioactivity near the start (Q zone) of the electrophoretograms. We have not found radioactivity in this zone in material from non-toxic nodular goitres from adults, but we have noted it in thyrotoxic glands from patients prepared for operation with methyl thiouracil and in Hashimoto's thyroiditis.

3. Conclusions

Four apparently distinct and reasonably well-defined biochemical abnormalities have been described in sporadic goitrous cretinism, but there are almost certainly others which will no doubt be revealed in time. Each of the four abnormalities already described is due to a particular defect in the synthesis of the thyroid hormones. All of these defects have a common result, namely, difficulty in producing sufficient effective thyroid hormone for the patient's metabolic needs. In some patients, such as those with a peroxidase or coupling enzyme defect, it is easy to understand why insufficient hormone is produced, but in others, such as those with a dehalogenase defect, it is more difficult to understand why this should be so. On genetic grounds it seems likely that different defects, which are almost certainly due to diminished activity of different specific enzymes, are unrelated, though there is evidence that diminished activity of one enzyme may indirectly affect another by producing hypothyroidism.

The familial tendency in sporadic goitrous cretinism and the occurrence of consanguinity of the parents were noted by nineteenth-century physicians. These observations have been fully confirmed. Twenty-five families, where siblings were affected, were responsible for 65 of 134 cases reported between 1943 and 1957. Twenty-one of these cases occurred in seven families in which the parents were definitely consanguineous. The influence of heredity is seen at its most marked in the family-group of tinkers studied in Glasgow. The high familial incidence and the frequent consanguineous marriages among the parents of the tinker cretins led us early in our studies to the conclusion that the biochemical anomaly responsible for the hypothyroidism in these patients was an inborn error of metabolism (Hutchison & McGirr, 1954). The information provided by the family-tree (fig. 1) and the more recent biochemical studies strongly support the opinion that the enzyme defect in these patients, namely, impaired dehalogenase activity, is genetically determined and that it is transmitted by a single incompletely recessive autosomal gene. The genetics

[2] Strips of filter-paper on which the proteins were separated by electrophoresis.—Ed.

of the other defects have not been worked out but the familial incidence suggests that they too have a genetic basis.

Similar enzyme defects account for some cases of sporadic goitre in young patients who present no other evidence of thyroid dysfunction (McGirr, 1960). These cases also show a notable familial incidence, and their defects almost certainly have a genetic basis. Some of the defects, such as diminished peroxidase activity, are obviously less complete in these cases. Taken in conjunction with the report by Pitt-Rivers, Hubble & Hoather (1957) of a derangement of the normal pattern of synthesis in non-toxic nodular goitres in adults, these findings encourage further research to establish whether or not inherited defects in hormone synthesis have a significant role in the pathogenesis of goitre.

It seems likely that defects at various levels of synthesis occur in varying degrees of severity, from the more or less complete, which produces goitrous cretinism recognizable in infancy, to the minimal, which comes to light only when specially sensitive tests such as the $M^{131}IT$ excretion test are used to detect it. It is not difficult to imagine that minor defects may occur, and account for goitres which develop later in life. Depending on their severity they may operate alone or they may require additional factors such as increased metabolic demands, iodine deficiency, or exogenous goitrogens to augment their effects (McGirr, 1960).

Implicit in a hypothesis which seeks to explain the pathogenesis of goitre on the basis of an enzyme defect is the belief that the goitre is a compensatory thyroid enlargement produced by increased thyrotrophin (thyroid-stimulating hormone) (TSH) secretion. Evidence of increased TSH, such as was found in the serum of one of the tinker cretins (McGirr *et al.* 1959a), is essential to maintain this hypothesis. If the enlarged thyroid fails to produce enough hormone, hypothyroidism as well as goitre results, as in sporadic goitrous cretinism, whereas if it compensates fully for its intrinsic inadequacy and produces enough hormone, goitre without other evidence of thyroid dysfunction results, as in sporadic goitre.

Females are only slightly more commonly affected than males by sporadic goitrous cretinism or sporadic goitre with established defects, the approximate ratio of female to male being 2:1. This contrasts with sporadic goitre, as it is usually met with in practice, which shows a marked predominance in females, who usually outnumber males by approximately 8:1. This difference in sex incidence is striking, and suggests that genetic factors, which affect men and women equally, cannot be the complete explanation of sporadic goitre (Trotter, 1959). It may be that sex factors particularly related to puberty, reproductive life and the menopause in females play a relatively insignificant role in the pathogenesis of goitre when severe defects in synthesis are inherited, but exert an important influence in patients with minor defects.

ACKNOWLEDGEMENTS

I wish to acknowledge my indebtedness to Dr James H. Hutchison, Royal Hospital for Sick Children, Glasgow, and Miss Elspeth W. Clement, Regional Physics Department, Glasgow, who have been associated with me in the clinical and laboratory aspects respectively of my own work.

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BIOSYNTHESIS OF THE THYROID HORMONES

ROSALIND PITT-RIVERS M.Sc. Ph.D. F.R.S.

National Institute for Medical Research
Mill Hill, London

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1. Thyroidal Iodine Trapping and Binding

The thyroid gland possesses a mechanism whereby it can concentrate iodide from the circulation. It is not unique in this respect; numerous workers (see Pitt-Rivers & Tata, 1959) have demonstrated iodide accumulation in the stomach, mammary gland and salivary gland and in the placenta (Klein, 1939). Logothetopoulos & Scott (1955) have found a concentration gradient of ^{131}I iodide between foetal and maternal blood in the rabbit and guinea-pig, but not in the rat. It has also been shown that certain marine algae can concentrate iodide, as such, from the surrounding medium (Jacques & Osterhout, 1938) or after oxidation to iodine (Shaw, 1959). Iodide concentration by *Fucus ceranoides* is inhibited by thiocyanate, perchlorate and nitrate (Klemperer, 1957) and therefore resembles the thyroidal iodide trap (Wyngaarden, Wright & Ways, 1952). Iodide concentration by *Nereocystis luetkeana* is also inhibited by thiocyanate, but not by perchlorate (Tong & Chaikoff, 1955). In the whole animal, only the thyroid and mammary glands have been shown unequivocally to bind iodine, and in vertebrates the thyroid gland alone possesses the mechanisms necessary to elaborate thyroid hormone.

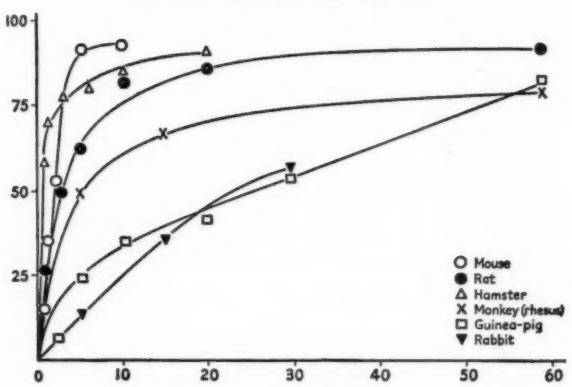
In recent years, three factors have been largely responsible for the advances that have been made in our knowledge of thyroidal iodine metabolism: firstly, the use of radioactive isotopes of iodine, principally ^{131}I, which has enabled workers to study the fate of iodine administered in a physiological range of doses; secondly, chromatographic analysis of ^{131}I-labelled compounds, which has revealed iodinated products in the thyroid and extra-thyroidal tissues that are present in extremely small amounts; thirdly, the discovery of two types of antithyroid drugs—those like thiocyanate and perchlorate, which inhibit the thyroidal iodide-concentrating mechanism or iodide pump, and those like thiourea and related compounds, which permit iodide concentration by the gland but prevent its incorporation into organic compounds. This discovery has allowed separate investigation of the iodide pump and the iodine-binding mechanism.

When iodide enters the thyroid, it is rapidly incorporated into organic linkage. Using autoradiographic techniques, Wollman & Wodinsky (1955) showed that, in the anaesthetized mouse, protein-bound ^{131}I ($PB^{131}I$) could be demonstrated in the gland within 11 seconds of intravenous injection of ^{131}I-labelled iodide ($^{131}I^-$). The $PB^{131}I$ increased when the thyroids were excised at longer time-intervals after radioiodide administration. These studies further showed that, at all time-intervals, the protein-bound iodine in the gland was in the colloid. At first the autoradiographs of the sections showed the radioactivity as thin rings at the edge of the colloid. Later the rings thickened and eventually spread throughout the colloid. From these observations and those of Doniach, Howard & Pelc (1953) it appears that thyroid hormone biosynthesis occurs entirely in the colloid, though this remains to be demonstrated by biosynthetic studies in vitro.

Recently Galton and Pitt-Rivers (unpublished) have found that the rate of thyroidal ^{131}I binding varies in different species. Groups of rats, mice, hamsters, guinea-pigs, rabbits and monkeys (rhesus) were lightly anaesthetized with ether or Nembutal, and ^{131}I was injected intravenously. The thyroid glands were removed at intervals from 15 seconds to one hour after ^{131}I injection and rapidly frozen and thawed several times to prevent further binding of ^{131}I. They were then chromatographically analysed for protein-bound ^{131}I and for $^{131}I^-$ by the method previously described (Pitt-Rivers, Galton & Halmi, 1958). In the smaller rodents it was found (fig. 1) that ^{131}I binding was exceedingly rapid; in the hamster 60% of the total ^{131}I in the thyroid was bound to protein within 15 seconds of the injection. ^{131}I binding was considerably slower in the monkey, guinea-pig and rabbit. The results shown here probably represent a somewhat slower rate of iodine binding than would occur in the normal animal, since anaesthesia has been shown to depress thyroid function in certain animals (Wase & Greenspan, 1953; Oyama, 1959). Other factors, such as dietary iodine and blood flow (see Söderberg, 1958), that affect thyroid function, were not determined. Nevertheless it appears that there is a species difference in the rate of iodine binding by the thyroid.

It is now generally believed that the first step in thyroid hormone biosynthesis is the formation of 3-monoiodotyrosine; in the chick-embryo thyroid, this has been shown to precede the appearance of di-iodotyrosine by about 18 hours

FIG. 1. Rate of Organic ^{131}I Binding in the Thyroid Glands in Different Species



Abscissae: time after ^{131}I injection (min.)
Ordinates: thyroidal protein-bound ^{131}I (percentage of total ^{131}I in thyroid)

(Trunnell & Wade, 1955; see also Willier (1955) for a discussion of hormone biosynthesis in the embryonic thyroid). In neonatal and subsequent life, the appearance of monoiodotyrosine is rapidly followed by that of di-iodotyrosine in many species studied. Later, thyroxine and 3:5:3′-tri-iodothyronine are found in the gland. These two are the only biologically active compounds synthesized by the thyroid and secreted into the blood. 3:3′-Di-iodothyronine and 3:3′:5′-tri-iodothyronine have also been detected in the thyroid (Roche, Michel, Wolf & Nunez, 1956), but the absence of any significant thyromimetic activity in either of these compounds suggests that they are biochemical curiosities rather than hormones (Gemmill, 1956; Stasilli, Kroc & Meltzer, 1959).

2. Thyroid–Pituitary Relationship

It has been known for many years that, for proper functioning, the thyroid gland is dependent upon the anterior pituitary secretion, thyrotrophic (thyroid-stimulating) hormone (TSH), and that the thyroid hormones themselves influence the rate of secretion of TSH. This reciprocal relationship provides a homeostatic mechanism which ensures adequate synthesis and secretion of thyroid hormone in changing circumstances.

In hypopituitarism, or after hypophysectomy, thyroid function is reduced to such a level that hypothyroidism results. It has been shown that hypophysectomy in the rat is followed by a marked decrease in thyroidal iodide trapping, incorporation of iodine into thyroglobulin (Taurog, Tong & Chaikoff, 1958a) and thyroid hormone secretion (Wolff, 1951). Administration of the anterior pituitary TSH to the hypophysectomized animal restores all the functions of the thyroid to normal levels (Taurog, Tong & Chaikoff, 1958b, and others). Further, the administration of the antithyroid drug propylthiouracil, which appears to stimulate TSH secretion by the pituitary, is followed by greatly elevated thyroid:serum (T:S) iodide ratio (McGinty & Sharp, 1946; Vanderlaan & Vanderlaan, 1947; Taurog, Chaikoff & Feller, 1947).

In man, the administration of TSH stimulates both thyroidal ^{131}I uptake and thyroid hormone secretion, the latter effect being more rapid than the former (Einhorn & Larsson, 1959). The administration of TSH to the normal animal will accelerate secretion of thyroid hormone from the gland and may cause colloid depletion and epithelial hypertrophy (Aron, 1929; Closs, Loeb & MacKay, 1932; Schockaert & Foster, 1932; Eitel, Krebs & Loeser, 1933; Brown-Grant, 1957). The vexed question, whether hyperpituitarism is the cause of hyperthyroidism, will not be discussed here (see Werner, 1955).

3. Hypothalamic Control of the Pituitary

The regulation of the secretion of trophic hormones from the pituitary by the hypothalamus has been the subject of a considerable number of investigations, and it has been shown that section of the pituitary stalk and electrolytic lesions in the hypothalamus are followed by depressed thyroid function (see Harris, 1955; Harris & Woods, 1957; Greer, 1957; D'Angelo & Traum, 1958). It has also been shown that, in the adrenalectomized animal, hypothalamic stimulation causes increased thyroidal activity (hormone secretion) though it does not do so in the intact animal; this is probably because the stimulus given to the thyroid by increased TSH secretion is counteracted by the increased adrenocorticotrophic hormone secretion (Harris & Woods, 1957), which is known to depress thyroid function (see Money, 1955; Pitt-Rivers & Tata, 1959).

The direct hypothalamic control of TSH secretion propounded by Harris (1955) and Brown-Grant (1957) has been questioned by D'Angelo (D'Angelo, 1958; D'Angelo & Traum, 1958), who found that destruction of a large part of the hypothalamus by electrocautery did not produce such a profound depression in thyroid function as was seen after hypophysectomy; they concluded that the thyroid hormone acts on the pituitary independently of the hypothalamus and that the thyroid–pituitary axis is not under neural domination.

Some recent experiments of Nikitovitch-Winer & Everett (1958, 1959) lend support to Harris's view. These workers showed that, in rats, pituitaries transplanted to the kidneys underwent marked degenerative changes accompanied by loss of function, but recovered normal morphological characteristics and showed evidence of restored trophic function after retransplantation to the median eminence. Functional improvement was shown by a rapid recovery of gonadotrophic activity, and was also demonstrable with respect to TSH, as shown by an increased 24-hour uptake of ^{131}I by the thyroid. These authors concluded that, in order to have normal morphology and function, the cells of the posterior pituitary must have direct access to hypothalamic stimulation, which they believed to be of a chemical nature and carried by the portal vessels of the pituitary.

Shibusawa, Nishi & Abe (1959) have described a neurohumour in the hypothalamus which stimulates thyrotrophin release from the pituitary; they call it thyrotrophin release factor or TRF. TRF has been detected by these workers in the anterior hypothalamus, blood, cerebrospinal fluid and urine of dogs and has been purified by column chromatography. Injection of TRF into intact rats and into those with hypothalamic lesions caused an increase in thyroidal T:S ^{131}I ratio and in thyroid weight. It no longer stimulated thyroid function in hypophysectomized animals (Shibusawa, Yamamoto, Nishi, Abe & Tomie, 1959). If these findings are confirmed, they will help to explain the nature of hypothalamic regulation of TSH secretion.

4. Autoregulation of the Thyroid

It has been suggested that the thyroidal iodide pump is to some extent controlled by the level of thyroid hormones in the gland itself, but from the evidence at present available this does not appear to be so (Halmi, 1957); it depends principally on the reciprocal regulation of TSH and thyroid hormone secretion. However, Halmi, Granner, Albert & Doughman (1959) have recently brought forward evidence that one of the thyroid hormones can influence the iodide pump by a different mechanism; they found that administration of tri-iodothyronine was more effective in inhibiting thyroidal iodide concentration in the rat than was hypophysectomy, although it had no effect on the T:S ratio of hypophysectomized animals. They therefore postulated that tri-iodothyronine, besides possessing a TSH-inhibitory action, has another effect, also mediated through the pituitary, whose nature is at present unknown.

5. Studies on Iodine Binding in Vitro

a. Thyroid

It was first demonstrated by Chaikoff and his colleagues (see Chaikoff & Taurog, 1949) that surviving thyroid slices

could concentrate $^{131}I^-$ and bind it to protein which, on hydrolysis, yielded di-iodotyrosine and thyroxine. These reactions were inhibited by anaerobiosis, inhibitors of cytochrome oxidase, and certain antithyroid drugs. Homogenization greatly reduced the iodine-binding power of thyroid tissue. Since these studies, a number of others have been made on organic iodine binding by thyroid and other tissues in vitro. Weiss (1953) and Fawcett & Kirkwood (1954) both found that the iodination of free tyrosine by thyroid homogenates was dependent on the presence of cupric ions, though this was not confirmed by Alexander (1959). The iodination of protein-bound tyrosine by sheep thyroid mitochondria and microsomes does not require added copper (Taurog, Potter & Chaikoff, 1955; Tong, Taurog & Chaikoff, 1957) but is stimulated by flavine co-factors. It is inhibited by cyanide, thiocyanate and thiouracil, but not by perchlorate. Serif & Kirkwood (1958) have found that a soluble system from beef thyroid depends upon H_2O_2 and added copper for its iodinating activity. It is inhibited by anaerobiosis. Both particulate and soluble iodinating systems are inhibited by catalase (Tong *et al.* 1957; Serif & Kirkwood, 1958).

The enzymes involved in iodine incorporation into thyroglobulin have not yet been isolated. The histochemical evidence of a thyroid peroxidase (Dempsey, 1944; De Robertis & Grasso, 1946) has received yet further experimental support than that quoted above: Alexander (1959) has shown that rat thyroid and salivary gland homogenates, in the presence of an enzymic H_2O_2-generating system, will incorporate $^{131}I^-$ into L-tyrosine, giving monoiodotyrosine and to a lesser extent di-iodotyrosine. This system was not found to require added copper and was inhibited by catalase, cytochrome oxidase inhibitors and certain antithyroid drugs (not by perchlorate).

The thyroid glands of several species have been shown to possess a proteolytic enzyme which breaks down thyroglobulin, thus making possible the secretion into the circulation of thyroxine and, to a lesser extent, tri-iodothyronine. This enzyme has been considerably purified by McQuillan & Trikojus (1953) by ammonium sulphate fractionation. Haddad and Rall (unpublished) have also purified the thyroid protease by column chromatography on cellulose derivatives and have obtained a preparation with about the same activity as that of McQuillan and Trikojus.

After proteolysis, the thyroid hormones pass into the circulation. In the normal animal, the free iodotyrosines do not leave the thyroid but are de-iodinated by the thyroid de-iodinase (Roche, Michel, Michel & Lissitzky, 1952). The iodide thus formed is presumably re-utilized for hormone synthesis. Roche and co-workers (Roche *et al.* 1952) found that the thyroid de-iodinase did not de-iodinate free thyroxine or tri-iodothyronine; however, Tata (unpublished) has shown that thyroid de-iodinase can de-iodinate thyroxine if the "protective" action of serum proteins is removed.

b. Other Tissues

Taurog, Potter, Tong & Chaikoff (1956) have shown that slices of rat lactating mammary gland and submaxillary gland can both form $PB^{131}I$ in vitro, though neither tissue was able to concentrate iodide. Slices of spleen and liver did not form $PB^{131}I$ to any significant extent; however, when these tissues were homogenized, higher yields of $PB^{131}I$ were obtained from mammary and submaxillary gland, and iodinating activity became manifest in spleen. Tong & Chaikoff (1955) have shown that both intact and homogenized tissue from the marine alga, *Nereocystis luetkeana*, can bind $^{131}I^-$; iodination by the homogenate yielded only monoiodotyrosine, but both iodotyrosines were obtained from hydrolysates of the intact tissue.

These results are of great interest in that they demonstrate the ability of certain animal and plant tissues to convert iodide to an organic form, even when their cells are disrupted. The physiological significance of these findings is not yet clear. In the whole animal (vertebrate) only the thyroid and mammary gland synthesize organic iodine, and in the latter this process stops at monoiodotyrosine (Potter & Chaikoff, 1956; Brown-Grant & Galton, 1958). A brief description of iodine organification in the dog's stomach (Howell & Van Middlesworth, 1957) awaits confirmation. Pitt-Rivers (unpublished) has studied the incorporation of $^{131}I^-$ into the protein of various tissues in intact and thyroidectomized rats. At no time could any binding be demonstrated in brain, heart, liver, kidney, spleen, skeletal muscle, adrenal, pancreas or thymus. Since spleen does not appear to bind iodine even in the absence of competition for iodide by the thyroid, the binding of ^{131}I by spleen homogenates does not represent a physiological process. It may be that, in the iodine-binding homogenates, the substrate and enzyme requirements of the embryonic thyroid are artificially brought together.

6. Iodine Binding in Invertebrates

Organic iodine has been found in an ascidian (Gorbman, 1941), in the marine alga cited above and in certain corals and sponges (see Roche & Michel, 1955). Di-iodotyrosine has been recognized for many years as a constituent of gorgonins, and traces of thyroxine have been detected in some, mainly in the exoskeleton. The biological significance of the iodotyrosines and of thyroxine in these species is not known. Whether they play a part in the iodine economy of the animal or whether they are slowly formed by chemical rather than enzymic reactions, because of environmental conditions, remains to be discovered.

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INFLUENCE OF THE THYROID ON THE CENTRAL NERVOUS SYSTEM

J. T. EAYRS* D.Sc. Ph.D.

Department of Anatomy
University of Birmingham

1 Neural manifestations of thyroid activity
  a Behaviour
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It is generally recognized that the thyroid gland plays an essential part both in the regulation of tissue metabolism and in the processes underlying maturation and differentiation. Thus evidence derived from both clinical and experimental sources leaves little doubt concerning the relationship between thyroid hormone and such factors as the utilization of oxygen and of vitamin A and its precursors, while studies which have shown the significance of the thyroid in the control of amphibian metamorphosis (see Allen, 1938) find a counterpart in the observation that, during mammalian development, thyroid hormone serves to regulate changes in form rather than to stimulate increase in size (Scow & Marx, 1945; Scow, Simpson, Asling, Li & Evans, 1949; Ray, Simpson, Li, Asling & Evans, 1950; Walker, Asling, Simpson, Li & Evans, 1952). Though few of these findings have been primarily concerned with nervous tissues it would be surprising were the nervous system to be excepted, and indeed many of the disorders now known to be attributable to thyroid dysfunction were recognized in early times by reason of their associated mental symptoms. Perhaps, however, because such disorders when adequately treated are rarely fatal the precise interrelationship between thyroid and nervous system has not been extensively studied in man. In the following account, therefore, emphasis has been laid on evidence derived from experimental rather than from clinical sources and, in view of the greater severity of symptoms of thyroid dysfunction when arising in the immature individual, on developmental factors.

1. Neural Manifestations of Thyroid Activity

a. Behaviour

The influence of the thyroid on the central nervous system (CNS) is evident from the changed demeanour of individuals suffering from disorders of the gland (see, for example, Means, 1948). Excessive thyroid secretion is associated with hyperexcitability, irritability, restlessness, exaggerated responses to environmental stimuli and emotional instability, symptoms which may be of sudden onset and, if untreated, ultimately lead to psychosis. The symptoms of myxoedema, on the other hand, make an insidious appearance and are generally characterized by listlessness, lack of energy, slowness of speech, reduced sensory capacity, impairment of memory and somnolence. The conditions of both hyper- and hypothyroidism arising in the adult, provided they are not of overlong duration, respond adequately to appropriate medication and are thus presumably related to abnormal cerebral metabolism. As Gregory (1956) has pointed out, the effects of thyroid hyperfunction may be regarded as secondary functional signs in so far as they represent an exaggeration of existing personality characteristics, whereas those of hypothyroidism are due to mental disorders primarily attributable to the endocrine dysfunction. This is particularly true of cretinism and juvenile myxoedema, in which the picture is complicated by the superimposition of defective maturation of a type to be considered in a later section.

The developmental aspects of the relationship between the thyroid and the CNS are well illustrated by differences in the response of mature and immature individuals to experimental treatments involving the thyroid. Marked changes in capacity for behaviour are not readily induced in adult laboratory animals as a result either of thyroidectomy or of giving thyroid hormone, and attempts by means of these procedures to implicate the thyroid in the mediation of such forms of behaviour as spontaneous activity (Hall & Lindsay, 1938; Brody, 1941a), the acquisition or performance of a conditioned response (Liddell & Bayne, 1926; Anderson, 1941) or discriminatory tasks (Kleitman & Teitelbaum, 1936; Thompson & Kenshalo, 1954) have yielded mostly negative or inconsistent results. Likewise, administration of thyroxine has been shown to cause little change in maze-learning ability (Mann, 1942) and, although Rickey (1925) claimed that thyroidectomy reduced the capacity of adult rats to learn a maze, these findings have not since been confirmed (Rockwell, 1931; Eayrs, unpublished). Thus, although inconsistencies among the experimental findings of different workers may well be due to variations in dosage (Crisler, Booher, Van Liere & Hall, 1933) or in factors of motivation (Denenberg, 1958; Denenberg & Myers, 1958), it seems clear that the effects of thyroid hormone on the behaviour of the mature experimental animal are relatively small.

A marked contrast is seen, however, when thyroid deficiency arises during the course of cerebral development. A retardation in the time of appearance of innately organized developmental landmarks, described both in man (Gesell, Amatruda & Culotta, 1936) and in the rat (Eayrs & Lishman, 1955), is followed by even more significant changes in adaptive behaviour, which can be interpreted in terms of a reduced susceptibility to the influence of the sensory input. Thus cretinoid[1] animals are found to be placid and more easily handled than the normal, quickly becoming habituated to a novel situation and readily learning a simple problem. They tend, however, to persist in the performance of an established habit in the face of small environmental changes to which the normal

* Henry Head Research Fellow of the Royal Society

[1] The terms "cretin" and "cretinoid", when used in relation to experimental animals, are used by the author to mean that an athyroid or hypothyroid state was induced during early infancy in otherwise normal young. However, see paper by Trotter, p. 92 of this number of the Bulletin, for a discussion of the meaning of the term "cretin".—ED.

animal will react, and hence, when faced with a more difficult task such as learning a maze, make more errors and spend less time in investigation as a result of each error (Eayrs & Lishman, 1955). More recent studies (Eayrs, 1960), using a closed field test of high reliability for the assessment of cerebral dysfunction (Rabinowitz & Rosvold, 1951), have confirmed the inferior capacity for behaviour of the rat made hypothyroid during infancy and have suggested in addition that a hard core of residual error towards the end of a period of learning is attributable to persistent repetition of the same error or group of errors combined with a tendency to respond to the dominant rather than to the less obvious features of the environment. The contrast between these observations and those relating to the behaviour of individuals made hypothyroid during adult life may be regarded as evidence of the influence of thyroid hormone as a factor affecting the maturation of the CNS. This is further supported by the demonstration of a close relationship between the performance of the hypothyroid rat and the age at which the thyroid was destroyed (Eayrs, 1960). The impairment has proved to be most severe in animals in which the thyroid is destroyed by radioiodine on the day of birth, and small though statistically significant when hypothyroidism is induced at 10 days of age. When the thyroid is destroyed at 24 days of age, the behaviour of the rats has proved to be virtually indistinguishable from that of the normal.

b. Excitability of Nervous Tissues

There is evidence that, as might be foreseen from the changes in behaviour, the excitable properties of nervous tissues are related to the activity of the thyroid. Lambert, Underdahl, Beckett & Mederos (1951) found that the speed of contraction and relaxation of muscle during the Achilles tendon reflex was increased in hyperthyroidism and reduced in myxoedema but, although they concluded from a study of the latent period that conduction in a reflex arc involving two neurones was little affected, their findings are consistent with the changes which occur in the chronaxia of peripheral nerve in both hyper- and hypo-thyroid states (Horsten & Boeles, 1949). Moreover, reaction time for the performance of a more complex response is increased by thyroidectomy (Brody, 1941b), indicating a central effect which may underlie the sluggish autonomic responses of cretinous children (Bower, 1957). Further evidence relating thyroid secretion to the level of excitability of nervous tissues is provided by the observation that, in the rat, the threshold and duration of seizures induced by electro-shock are decreased by giving thyroxine and increased by thyroidectomy, both procedures accelerating post-ictal recovery (Woodbury, Hurley, Lewis, McArthur, Copeland, Kirschrink & Goodman, 1952). Similar findings have been recorded in the guinea-pig (Gerlich, 1949) and rabbit (Domino & Minz, 1953).

Changes in central nervous activity as a result of thyroid dysfunction are likewise reflected in the electroencephalograph (EEG), Ross & Schwab (1939) finding a good positive correlation between thyroid activity and the frequency of the α rhythm. The records of hyperthyroid patients have been found to present features resembling those seen in epilepsy (Condon, Becka & Gibbs, 1954), and in chronic hypothyroidism changes occurring in the EEG include a reduced amplitude, disappearance of the α rhythm and of regional differences in rhythmic activity, and absence of the normal response to photic stimulation (Bertrand, Delay & Guillain, 1938). These abnormalities, although they may not all appear at the same time in the same individual (Scheinberg, Stead, Brannon & Warren, 1950), are strikingly similar to those recently observed by Bradley, Eayrs & Schmalbach (1960) in the rat made hypothyroid at birth but not in animals made hypothyroid later in life. They also appear to resemble those described by Topper (1951) in the EEG of cretinous children as suggestive of "diffuse cerebral dysfunction."

c. Cerebral Structure

Although the administration of thyroid hormone would appear to exert a relatively small influence on cerebral structure (Tusques, 1956), there can be no doubt concerning the profound effect of its deficiency. In man, Mott (1917) recorded depletion of Nissl substance in the cortical neurones of myxoedematous individuals, while others have described severe changes in the brains of cretins amounting to cerebral hypoplasia (Marie, Trétiakoff & Stumfer, 1920; Lotmar, 1929, 1933) and chronic vascular disturbance (Benda, 1947). More recently a series of quantitative studies, based largely on rats made hypothyroid from birth by giving daily the goitrogen methylthiouracil (Hughes, 1944), or by a single injection of radioiodine (Goldberg & Chaikoff, 1949), has revealed something of the manifold nature of the structural sequelae of thyroid deficiency arising during the course of development. Briefly summarized, these include the features discussed below.

i. Gross Morphology of the Brain

The weight of the brain is significantly reduced and its shape altered in so far as the infantile characteristics of a cerebrum, which is disproportionately wide in relation to its length, are retained (Eayrs & Taylor, 1951).

ii. Cortical Histology

The perikarya of the cortical neurones are smaller than those of normal litter-mates and are at the same time more closely aggregated (Eayrs & Taylor, 1951). The increased packing density of the neurones is not solely due to their reduced size but is associated with a significant impairment to the development of cell processes. Thus the myelination of axons is retarded (Barrnett, 1948) and the density of the axon network is reduced throughout the cortical thickness, and particularly in the region occupied by the plexuses formed by thalamic sensory projections (Eayrs, 1955). Similarly, the growth of dendrites is impaired, and although the number of dendrites primarily arising from the perikaryon is little affected, both the amount of branching and peripheral extension of these processes is considerably reduced, while the form of decay in the density of the dendritic field with distance from the centre of the perikaryon departs from that seen in the normal animal. Analysis of the changes in cortical structure that normally occur with advancing age suggests that the developmental abnormalities seen in hypothyroidism represent a distortion rather than a retardation of growth (Eayrs & Goodhead, 1959). Their effect represents a considerable reduction in the probability of axo-dendritic interaction between neurones which, assessed by the method described by Uttley (1955), amounts to the considerable factor of about 80% in the 24-day-old rat.

iii. Vascularity

Vascular disturbance of a type likely to impair cerebral function, such as increased capillary permeability (Lange,

1944), reduction in the extent of the capillary bed (Zondek, Michael & Kaatz, 1941) and degenerative changes (Kountz, 1951), have been reported in many of the tissues of the body, and Benda (1947) has attributed the mental impairment of cretinism to the impact of such changes on cerebral development. Observations based on experimental material are certainly consistent with this view. In the rat made hypothyroid from birth there occurs a marked reduction in the extent of the vascular bed which is associated with capillary enlargement, the cerebral tissues being served by a relatively small number of apparently dilated capillaries (Eayrs, 1954). In such a system not only will a larger volume of excitable tissue be placed at a nutritional disadvantage by reason of its remoteness from its blood supply, but the capillary surface available for metabolic exchange must also be appreciably reduced.

iv. Enzymology

Studies of the effects of thyroid hormone on enzyme activity in the nervous system have lagged behind those made on other tissues of the body and have yielded, on the whole, negative results. Thus Markoff (1935) and Rossiter (1940) failed to record in rats given thyroxine any changes in the activities of cytochrome oxidase and of pyruvic and succinic oxidases respectively. Lately, however, the studies of Hamburgh & Flexner (1957) have indicated a measure of specificity in the relationship between thyroid hormone and enzyme activity in the developing brain. In conformity with the observations of earlier workers, no significant effect was observed in relation to cytochrome oxidase or aldolase, but on the other hand a significant depression was recorded in the activities of both cholinesterase and succinic dehydrogenase.

2. Reversibility of Effects of Thyroid Dysfunction

While there is little doubt that, when the condition has arisen in the adult, the various mental sequelae of thyroid dysfunction can be reversed on restoration of the euthyroid state, the efficacy of replacement therapy on abnormalities resulting from thyroid deficiency during development is less well established. The view has been widely held that the cerebral changes seen in congenital hypothyroidism are entirely due to thyroid deficiency (see Kerley, 1936; Wilkins & Fleischmann, 1941) and that their severity and reversibility are directly related to early diagnosis and treatment (Aldrich, 1936; Means, 1948). The finding of Goodkind & Higgins (1941), for instance, to the effect that children diagnosed as suffering from juvenile myxoedema responded better to treatment than did cretinous individuals, is consonant with this view. On the other hand, while it is generally agreed that the intelligence quotient of most cretins is destined to remain subnormal (Kimball & Marinus, 1930; Rolleston, 1934; Brown, Bronstein & Kraines, 1939), there are cases on record in which medication has resulted in the attainment of a satisfactory mental status, and many have been unable to equate this variability of response with the adequacy of treatment. Lewis (1937), for example, has recorded cases in which subjects receiving both early and continuous medication remained grossly defective, while others showing physical signs of hypothyroidism achieved intelligence quotients within the normal range, and comparable findings have since been reported by Radwin, Michelson, Berman & Kramer (1949). Bruch & McCune (1944) concluded that the intellectual inferiority of hypothyroid children is sometimes due to a primary defect in cerebral development rather than to a lack of thyroid hormone without, however, defining the extent to which such irreversible changes might be attributed to thyroid dysfunction. One possibility is that they are caused by thyroid deficiency during the intrauterine period of development (Kimball & Marinus, 1930; Wilkins, 1938). An alternative hypothesis, suggested by Tredgold (1929) and later supported by Goodkind & Higgins (1941), implies that the irreversible defects shown by many cretins are the outcome of some congenital abnormality arising at the same time as—but not necessarily related to—the primary hypothyroidism. This view has been criticized by Brown *et al.* (1939) on the ground that there is frequently no history of primary amentia in the families of cretins not responding to treatment.

There are obvious difficulties in the way of resolving such a problem by analysis of clinical material. The picture is often complicated by uncertainties centred about the correctness of initial diagnosis and the thoroughness with which the prescribed medication has been carried out in the absence of supervision, but the recent comprehensive study of Smith, Blizzard & Wilkins (1957) strongly supports the view that the time of onset and degree of hypothyroidism, and the age at which medication is initiated, are important factors determining the severity and reversibility of the mental symptoms. Thus hypothyroidism acquired after the second year of life was found to engender little permanent defect, mild cretinism could be treated satisfactorily at a later age than athyroidism, and in athyroid individuals early treatment proved considerably more effective than late. At the same time, a considerable proportion of such patients did not respond to treatment and, while the reason for this remains open to conjecture, recent experiments which have given results strikingly similar to those of Smith *et al.* (1957) have helped to throw some light on the problem.

The nervous system of the rat is exceedingly immature at birth and in this respect is comparable with that of man during gestation. It might therefore be expected that a study of the effects of replacement therapy on neonatally thyroidectomized animals would indicate the extent to which the cerebral defects associated with thyroid deficiency uncomplicated by congenital factors were irreversible. Until recently the limited evidence from anatomy and behaviour seemed to suggest that these defects could be rectified by replacement therapy. Thus the density of the axon network may be to some extent restored by giving thyroid hormone (Horn, 1955), and the ability of cretins which are given thyroxine to learn a maze presenting multiple environmental cues was found not to differ from that of normal litter-mates (Eayrs & Lishman, 1955). A somewhat different picture has emerged, however, after further examination of the problem. Bradley *et al.* (1960) have found that medication only partially rectifies the abnormal character of the EEG in the cretinoid rat, and studies of the behaviour of such individuals in a more difficult situation than was previously used have left little room for doubt that the reversibility of the effects of thyroidectomy carried out at birth is directly related to the age at which therapy is begun (Eayrs, unpublished). If started before the 10th–15th day of age, recovery appears to be complete, but if delayed until the 24th day it is only partial. These findings are consistent with those of Hamburgh & Flexner (1957), who showed that the activity of succinic dehydrogenase in the cerebral cortex of the cretinoid rat was restored to normal

only if treatment was begun before the 10th day of life. Thus the experimental evidence, although not eliminating the possibility that congenital cerebral abnormalities can account for the failure of some cretins to respond to thyroid therapy, does show that irreversible changes can arise purely as a result of thyroid deficiency and so supports the view that the frequent failure of medication to achieve greater success in man is due to the incidence of a developmental defect resulting from thyroid deficiency in utero.

3. Nature and Significance of Relationship between Thyroid and Nervous System

a. Specificity of Relationship

We may now inquire whether the influence of the thyroid on nervous structure and function is selectively mediated or is the outcome of some indirect or non-specific relationship.

There is good reason to believe that, in pre-metamorphic amphibia at least, thyroid hormone acts directly on nervous tissues. Kollros (1943), for instance, showed that the local application of thyroxine to the brain of the frog tadpole caused an advance in the time of appearance of the corneal reflex so discrete as to be obtainable unilaterally by asymmetrical treatment. Furthermore, Weiss & Rossetti (1951) have shown that thyroid hormone can act differentially in so far as, under its influence, primitive integrative systems regress while there occurs at the same time a proliferation of elements believed to be concerned with the co-ordination of limb movements. This apparently selective effect on the development of those neural structures destined to play a significant part in post-metamorphic life has since been confirmed (May & Mugard, 1955; Tusques, 1956). That such changes are not secondary to modifications in somatic structure is shown by their elicitation by doses of hormone inadequate to induce general metamorphosis and by the failure of artificially induced peripheral changes to give rise to corresponding modifications in the CNS. Nor would these appear to be due to the effect of metabolic processes, since dinitrophenol, which increases metabolism, does not induce metamorphosis, whereas acetylated thyroxine, which stimulates metamorphosis, does not increase metabolism (Swingle, Helff & Zwemer, 1924).

Although the process of maturation in mammals may, in many respects, be regarded as the counterpart of metamorphosis, there is little direct evidence to suggest so specific a relationship between thyroid hormone and the developing nervous system as is found in amphibia. It is true that the administration of thyroxine to the new-born accelerates maturation without a corresponding increase in size, thus producing individuals which appear as caricatures of the adult, and that these changes are associated with an advance in the time of appearance of innately organized patterns of behaviour (Tusques, 1956). However, the results of preliminary experiments have failed to show that the capacity for adaptive behaviour of the individual given thyroxine during maturation is in any way more advanced than that of the normal (Eayrs, unpublished) and, apart from the demonstration of structural changes in the cerebellum, an organ known to be particularly susceptible to teratogenic procedures, there is no histological evidence to show in the mammalian nervous system a relationship comparable with that seen in amphibia.

The extent to which the impaired cerebral development associated with hypothyroidism may be attributed to a direct, rather than a secondary, influence upon nervous tissues is by no means resolved and it seems likely that both modes of action are involved. One factor which may exert an indirect effect is a disparity in growth between brain and cranium, attributable to a failure in endochondral ossification at the base of the skull (Dye & Maughan, 1929). Similar changes occur after hypophysectomy during infancy, which may be associated with herniation of the brain through the foramen magnum (Walker, Simpson, Asling & Evans, 1950). Such a developmental defect is consistent with the previously described changes observed in the shape of the brain of the hypothyroid rat and might be expected to give rise to an increase in intracranial pressure which, by restricting the return of venous blood from the brain, could be responsible for the capillary enlargement seen in the vascular bed and at the same time predispose the cerebral tissues to stagnant anoxia. It has so far not been possible, however, to demonstrate the increase in intracranial pressure which might be expected in these circumstances (Eayrs, 1956).

In support of the view that the effects of hypothyroidism are indirectly mediated, Tusques (1956) has emphasized the part played by malnutrition in retarding cerebral maturation, and the changes arising in vascular pattern give reason to believe that such may well be a prominent causal factor (Eayrs, 1959). On the other hand, the effects of chronic inanition differ in many respects from those of hypothyroidism (see Eayrs, 1959) and it is difficult to fit the findings of Hamburgh & Flexner (1957) relating to enzymic changes into a picture in which only nutritional factors are involved. Moreover, while many of the morphological changes arising in the absence of thyroid hormone may, in part at least, be due to an impaired capacity to synthesize protein (Hamburgh, 1955), the administration of substances known to stimulate protein anabolism and to restore the growth of the thyroidectomized individual, e.g., methylandrostenediol (Salgado & Selye, 1954); anterior pituitary growth hormone (Scow & Marx, 1945; Ray *et al.* 1950), neither repairs nor prevents the development of defects attributable to early hypothyroidism (Eayrs, communication to the Seventh International Anatomical Congress, April 1960, New York).

b. Mode of Action of Thyroid Hormone on the Central Nervous System

Little can be said with certainty concerning the way in which thyroid hormone exerts its influence on the excitability, functioning or development of nervous tissues. In the adult it seems likely that many of the reversible effects of thyroid disorder may be attributable to the malfunction of an otherwise normal nervous system engendered by such factors as a disturbed electrolyte distribution (Timiras & Woodbury, 1956), oxygen and glucose metabolism, and inadequate cerebral circulation (Scheinberg *et al.* 1950). The observation that dinitrophenol fails to restore cerebral activity in the hypothyroid individual to normal (Hall & Lindsay, 1938; Woodbury, 1954) suggests, however, that the impairment is not secondarily due to a reduction in basal metabolic rate or body-temperature alone.

The explanation of the morphogenetic changes associated with hypothyroidism is even less well defined. Reference has already been made to some of the factors which might be expected to exert a general influence on cerebral development, but none of these would seem adequate to account for what is probably the most significant of the several abnormalities

observed—a hypoplastic neuropil. While, as suggested by the early work of Hammett (1922), a reduced growth of cell processes may reflect a reduced capacity to synthesize protein within the perikaryon, too little is known about the factors which control their branching to permit of more than speculation concerning the impairment seen in the cretin. As shown by Gersh & Catchpole (1949), however, growth is frequently associated with a reduction in the viscosity of the intercellular matrix which, in the CNS, appears to be a muco- or glycoprotein (Goodhead, 1957). The presence of highly polymerized mucoid infiltrations of similar composition elsewhere in the body in hypothyroidism (Brewer, 1951) suggests that an increased viscosity of the ground substance may provide an unsuitable medium for the initiation of branching and the growth of processes. No experimental evidence is available to support this possibility though Horn & Hess (1958) have suggested that the ground substance may be increased in concentration as a result of thyroid deficiency.

c. Functional Significance

It is clearly difficult in the present state of knowledge concerning the relationship between cerebral structure, metabolism and function to link up so wide a variety of abnormalities into a comprehensive explanation of the nature of the impact of the thyroid on the CNS. From a functional point of view the reduced probability of interaction between neurones associated with a hypoplasia of the neuropil may be invoked to explain the changes which occur both in the electrical activity of the brain and the behaviour of the cretin. Thus in the light of the suggestion that the slow rhythms of the EEG may be due to intracortical stimulation through axo-dendritic synapses (Clare & Bishop, 1955), the absence of slow waves and the reduced amplitude of the trace in the cretin may reflect the reduction in the density of the dendritic field and of the number of axons available to form synaptic connexions (Eayrs, 1960). Likewise, the behaviour of the cretin is such as might be predicted to result from a reduced connectivity (see Eayrs, 1955; Eayrs & Lishman, 1955), on the reasonable assumption that the cerebral cortex responds in accordance with the principles of conditional probability (Sholl & Uttley, 1953) and has much in common with that arising when neuronal interaction is reduced by other means, e.g., neonatal anoxia (Becker & Donnell, 1952) or administration of nitrous oxide (Steinberg, 1956). Such considerations will clearly need amplification, however, if they are to provide a basis for explaining the enduring nature of the developmental impairment of congenital hypothyroidism, possibly in terms of the enzymic aspects of cerebral function and of changes in the chemical factors underlying the synaptic relationship between neurones.

4. Summary and Conclusions

The foregoing survey of evidence drawn from clinical and experimental sources has shown that the influence of the thyroid on the nervous system is reflected in cerebral morphology, in the electrical activity of the brain and in innately organized and adaptive behaviour. By reason of their impact on developmental factors, the effects of thyroid hypofunction are less severe when arising in the adult than in the maturing individual in which a permanent impairment may be due both to a direct, though as yet undefined, relationship between thyroid hormone and neural maturation, and to the secondary influence of thyroid deficiency on other tissues. Evidence is still lacking concerning the factors which are responsible for the permanent or semi-permanent nature of the developmental abnormalities but it is increasingly clear that both their severity and intractability are related to the age of onset of the thyroid disorder.

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THYROID-STIMULATING HORMONE

H. D. PURVES M.Sc. M.B. Ch.B.

D. D. ADAMS M.B. Ch.B.

New Zealand Medical Research Council
Endocrinology Research Department
University of Otago Medical School, New Zealand

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The first demonstration of the action of the thyroid-stimulating hormone (TSH) was made by Smith & Smith (1922), who showed that, in hypophysectomized tadpoles, injections of anterior lobe tissue from the bovine hypophysis would repair the atrophy of the thyroid and stimulate it to secrete hormone. That thyroid secretion was stimulated was evident from the induction of metamorphosis, which was known from the work of Gudernatsch (1914) to be dependent on thyroid activity. The experiments of P. E. Smith, by demonstrating that the action of thyrotrophin was not species-specific, and that it was effective only if administered by parenteral injection, blazed the trail for future investigators. Subsequently, Smith (1926), after developing a technique for hypophysectomizing rats, was successful in demonstrating the action of thyrotrophin in the hypophysectomized mammal.

1. Site of Production

TSH is secreted by the anterior lobe of the pituitary gland and has not been shown to be produced in any other site. From the work of Smith & Smith (1923), Azimov & Altman (1938), and Smelser (1944), it is evident that TSH is especially concentrated in the basophil zone of the bovine anterior lobe and that its distribution differs from that of all other anterior lobe hormones. Gonadotrophin and corticotrophin are less sharply localized to the basophil zone, while somatotrophin and prolactin are found in higher concentration in the acidophil zone. TSH must therefore be formed and secreted independently of the other pituitary hormones.

Recent developments in pituitary cytology indicate that the basophil cells of the pars anterior are not a homogeneous group but comprise at least three different cell types which can be distinguished from each other by differences in the appearance of the cells and by their responses to certain staining reactions. One of these specific basophil cell types appears to be concerned exclusively with the secretion of TSH. Purves & Griesbach (1951) proposed the name "thyrotroph" for the TSH-secreting cells. Cytological studies have made it possible to identify thyrotrophs in the pars anterior of a number of vertebrate species: rat (Purves & Griesbach, 1951); bat (Herlant, 1956); guinea-pig (D'Angelo, 1955); *Xenopus* (Cordier, 1953); amphibia and bony fish (Herlant, 1954). In these species the specific granules of the thyrotrophs are stainable by Gomori's aldehyde fuchsin (Gomori, 1950), and the thyrotrophs are therefore β cells in the restricted sense in which the term is used by Romeis (1940) and by Halmi (1950). The thyrotrophs are not in all species the only basophil type with granules stainable by the aldehyde fuchsin method, so that the terms "β cell" and "thyrotroph" cannot be equated. This is notably true of the human hypophysis, in which the thyrotroph has not yet been definitely identified.

Centrifugation experiments with tissue homogenates dispersed in sucrose solution show that TSH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) sediment with the basophil granules (Herlant, 1952; McShan, Rozich & Meyer, 1953; Brown & Hess, 1957). This and a parallelism between hormone content and granule content in experimentally modified pituitaries indicate that the hormones are contained in the granules which are the storage form of the cells' characteristic secretions. Chemically the granules of basophil cells are composed of glycoprotein, as is evident from their strong reaction with the periodic acid-Schiff histochemical test (McManus, 1946) for carbohydrate. There is therefore some reason for supposing that TSH and also LH and FSH are glycoproteins.

2. Actions of Thyroid-Stimulating Hormone (TSH)

TSH accelerates the metabolism of iodine by the thyroid gland. The immediate effect is an acceleration of the release of preformed hormones stored in the form of colloid. In small animals an elevation of the thyroxine content of the blood is evident within 30 minutes of an injection of TSH, and the effect of a single injection continues for only about three hours. Continuous or repeated stimulation by TSH results in a progressive depletion of the colloid content of the gland and a corresponding reduction in iodine content.

The reduction of the colloid content is evident on histological examination as a reduction in the proportion of colloid in the gland and a thinning of its substance. Prolonged or repeated stimulation also causes hypertrophy of the cells, changing the normally flat-celled epithelium to a cuboidal or columnar condition. With mild stimulation the increase in the mass of the cells is balanced by the loss of colloid so that there is little net increase in the weight of the gland. With stronger stimulation an increase in the number of cells by mitotic division occurs and the combined effects of hyperplasia and increasing cellular hypertrophy result in marked increases in the gland mass. Vascularity is strikingly increased and appears to be out of proportion to the energy requirements of the gland. This rich blood supply is required to meet the need of the gland for iodine.

With the aid of radioactive iodine, it is possible to demonstrate an increase in the rate of iodine assimilation by the stimulated gland. In thyroids in which the formation of organic iodine compounds is inhibited by an antithyroid drug, the ability of the gland to accumulate inorganic iodide can be shown to be strongly affected by TSH stimulation (Vanderlaan

& Vanderlaan, 1947). The action of TSH in increasing this iodide-trapping activity of the thyroid is subject to a distinct lag in comparison with its prompt action in accelerating the rate of discharge of hormone. Halmi, Spirtos, Bogdanove & Lipner (1953) found that trapping of iodide in the rat thyroid was increased by a single injection of TSH, with a latency of 5–6 hours, and reached its maximum at 24–48 hours. It seems that TSH affects iodide trapping in two ways—by lowering the iodine content of the gland and by a direct stimulating action on the cells. There is also evidence that the iodine supply to the gland affects the iodide-trapping mechanism independently of TSH action.

The acceleration of the rate of assimilation of iodine into organic compounds that follows a single injection of TSH is also subject to a distinct lag. In the human subject, Stanley & Astwood (1949) found an increased uptake of ^{131}I within eight hours of TSH administration. The fact that the increased discharge of hormone from the thyroid in response to TSH stimulation precedes the increased iodine assimilation is evident from the consistent fall in the total iodine content of the gland that follows TSH injection (Closs, Loeb & MacKay, 1932; McCullagh & Stimmel, 1935).

3. Assay

The observation of the histological changes produced in the thyroid of the hypophysectomized mammal serves for the identification of TSH. The cellular stimulation is directly visible, and from the observed loss of colloid a discharge of hormone can be inferred to have taken place. If the full histological response is observed, any further tests to characterize the hormone would seem unnecessary, but the measurement of an increased iodide trapping or ^{131}I assimilation can be carried out in conjunction with the histological test.

For the quantitation of the hormone in pituitary extracts, a large variety of tests involving different responses in different animals have been used. Of major importance, however, are the assay methods with sufficient sensitivity to detect TSH in body-fluids. A selection of those methods which have proved themselves useful in this respect is given in Table I.

TABLE I. Methods Used in Assaying TSH in Body-Fluids

Method	Reference
Measurement of acinar cell height in thyroxine-injected guinea-pigs	Purves & Griesbach (1949)
Measurement of acinar cell height in starved tadpoles	D'Angelo & Gordon (1950)
Thyroid ^{131}I uptake in thyroxine-injected guinea-pigs	Henry & Bloche-Michel (1955)
Thyroid ^{131}I uptake in iodocasein-treated mice	Querido, Kassenaar & Lameijer (1953)
Thyroid ^{32}P uptake in the chick	Kriss & Greenspan (1954)
Thyroid ^{131}I discharge in thyroxine-injected chicks	Gilliland & Strudwick (1953)
Release of ^{131}I into blood of thyroxine-injected guinea-pigs	Adams & Purves (1957)
Release of ^{131}I into blood of thyroxine-treated mice	McKenzie (1957)
Increase of weight of thyroid slices in vitro	Heideman, Bakke & Lawrence (1959)

4. Units

The International Unit (i.u.) is 13.5 mg. of the standard substance (Mussett & Perry, 1955) and was so defined to make it equal to the United States Pharmacopeia (USP) unit, which is 20 mg. of the USP "Thyrotropin Reference Substance". Both preparations consist of a mixture of one part of ox anterior lobe extract with 19 parts of lactose. The Junkmann-Schoeller unit, which was widely used before the introduction of these standards, lacked precise definition but is considered to have been about one-tenth or one-twelfth of an International Unit.

5. Chemical Properties and Attempts at Purification

The anterior lobes of ox pituitaries (approximately 0.02 i.u./mg. dry wt.) constitute the usual starting material for the preparation of TSH. Fractional precipitation of aqueous extracts by salt, ethanol or acetone raises the potency to about 1 i.u./mg. and permits the removal of most of the other hormones. Adsorption by a cation-exchange resin (Pierce & Nyc, 1956), by carboxymethylcellulose (Condliffe & Bates, 1956) or by diethylaminoethylcellulose (Condliffe & Bates, 1957) have been used to effect further purification and have given preparations containing 20 i.u./mg. Even the most potent preparations obtained appear to be heterogeneous (Pierce, Wynston & Carsten, 1958). The apparent isoelectric points and sedimentation constants are different for materials prepared by different methods. Potent TSH preparations contain protein-bound carbohydrate, but the carbohydrate content does not appear to increase with increasing purity. In its solubility in high concentrations of salts or ethanol, its failure to be precipitated by trichloroacetic acid, and its ability to penetrate, albeit slowly, through dialysis membranes (Fels, Simpson, Sverdrup & Evans, 1952), TSH behaves like a protein of low molecular weight.

6. Relation of TSH to Exophthalmos

Pituitary extracts rich in TSH produce exophthalmos in experimental animals, suggesting that exophthalmos may be the result of an extra-thyroidal action of TSH. Recent studies indicate that TSH alone is not responsible for the production of exophthalmos, since it may be purified to a point where it no longer produces this effect (Dobyns & Steelman, 1953; Smelser & Ozanics, 1954). TSH possibly acts synergistically with another factor to produce exophthalmos, and Smelser & Ozanics (1955) suggest that the second factor may be corticotrophin. These observations are of great interest as concerning the mode of production of exophthalmos by the administration of pituitary extracts to experimental animals, but their relevance to the problem of clinical exophthalmos in human thyroid disorders is doubtful. In primary myxoedema high levels of TSH are found in the blood, and the pituitary must be assumed to be secreting TSH at its maximum rate, yet in such cases exophthalmos does not develop. Moreover, large amounts of TSH have been administered to patients with myxoedema without producing exophthalmos or any other signs of extra-thyroidal action (Kyle, Welham, Doolan & Schaaf, 1954). It seems probable that clinical exophthalmos differs in its nature and its cause from that produced by pituitary extracts administered to experimental animals.

7. TSH Content of Serum from Euthyroid People

The human thyroid is relatively sensitive to TSH, and the level of TSH in the blood is correspondingly low. From the work of Greer & Shull (1957) it appears that the human pituitary may secrete not more than 2 i.u. of TSH/day. The average stay of TSH in the human circulation has not been measured. In the rat, it appears from the results of Levey & Solomon (1957) that exogenous TSH injected into the circulation does not enter the erythrocytes and that its average stay in the plasma is about 20 minutes. If we assume that in man 2 i.u. of TSH are secreted daily at a steady rate into 2.75 l. of plasma, and have an average stay in the plasma of 20 minutes, the equilibrium concentration of TSH in human plasma would be 0.01 unit/l. or 0.01 milli-unit/ml. Adams (1958) obtained no significant responses with serum from normal subjects, using an assay method capable of detecting 0.1 milli-unit/ml. Using a pool of 2 l. of serum from healthy individuals and concentrating the TSH by a two-stage process, in which carboxymethylcellulose absorption was followed by a fractionation using acetone, Purves, Adams & Sirett (unpublished observations) have obtained satisfactory responses with the Adams and Purves method of assay (see Adams & Purves, 1957), which, after allowing for the recovery (55%), indicate that the TSH content of the pooled serum was 0.012 milli-unit/ml. This low level emphasizes the difficulty of detecting TSH in the serum or plasma of a single individual except in conditions in which the secretion of TSH is many times more rapid than normal.

8. TSH in Hypothyroidism

An inadequate supply of thyroid hormone stimulates the secretion of TSH to a remarkable extent. The TSH content of the blood is raised to a level which is detectable by many assay methods. Quantitative measurements by Gilliland & Strudwick (1956), Di George, D'Angelo & Paschkis (1957), and Adams (1958) indicate plasma levels of about 2 milli-units/ml. in cretinism, in adult myxoedema, and in hypothyroidism induced by surgical thyroidectomy. This level is approximately 100 times the normal level in euthyroid individuals. There is evidence that in long-standing thyroid deficiency the pituitary itself is affected, since the blood TSH levels tend to be lower where the condition has been present for a long time.

As would be expected, TSH is not detectable in the blood of patients suffering from hypopituitarism (Gilliland & Strudwick, 1956).

9. Graves's Disease

In primary Graves's disease, or toxic diffuse goitre, there is a general enlargement of the thyroid gland, and obvious signs of hyperfunction are present. The histological changes indicative of thyroid stimulation affect the gland diffusely. These appearances indicate the presence of a humoral thyroid-stimulating agent. Such an agent can be demonstrated in the blood of at least some patients, and the failure to find it in all cases can be ascribed to the lack of sensitivity of the methods used. The position in secondary Graves's disease, or toxic multinodular goitre, is more obscure, since the histological appearance of the gland is not uniform, and active areas are interspersed with inactive areas. The question of a circulating thyroid-stimulating agent in this condition must be further investigated. Both these conditions must be distinguished from hyperthyroidism due to autonomously functioning thyroid tumours, in which condition the involution of the remaining normal thyroid tissue shows that TSH secretion is suppressed, as would be expected, by excess circulating thyroid hormone.

The characteristic feature of the thyroid activity of Graves's disease is that it is not regulated. Even the administration of 2 mg./day of tri-iodothyronine, an amount at least 20 times as much as the normal human requirement, does not suppress thyroid activity in these cases. The presence of non-suppressible thyroid activity does not always lead to excessive secretion of hormone, since Werner (1955) has shown that euthyroid individuals with the eye signs of Graves's disease have non-suppressible thyroid activity. In the majority of cases, however, the non-suppressible thyroid activity supplies more than the normal requirement of thyroid hormone, so that hyperthyroidism is present.

In addition to the non-suppressible thyroid activity, hyperthyroid patients possess a normal TSH-secreting mechanism which is in abeyance so long as the hyperthyroid condition persists. When these patients are treated with sufficient doses of goitrogenic drugs to render them hypothyroid, a marked progressive thyroid enlargement follows which must be ascribed to the secretion of TSH by the pituitary in response to a fall of the thyroid hormone concentration in the blood below the euthyroid level.

Positive responses indicating the presence of a thyroid-stimulating agent in the blood of patients with Graves's disease have been obtained by a number of investigators using different assay methods (Purves & Griesbach, 1949; D'Angelo, Paschkis, Gordon & Cantarow, 1951; Asboe-Hansen, Iversen & Wichmann, 1952; Gilliland & Strudwick, 1956). In general the responses given to the blood of patients with marked or progressive exophthalmos have been stronger than those given to the blood of patients in whom eye signs were slight. These investigators did not find a thyroid-stimulating agent in every case; but, in view of the low TSH content of normal human serum, it is evident that a thyroid-stimulating agent might be present in amounts capable of producing hyperthyroidism and not be consistently detected by the methods then in use. The significance of these positive results has, however, been altered by the discovery that the thyroid-stimulating agent present in the blood of such patients is not normal TSH. It differs from TSH in exerting a prolonged action on the thyroid of the guinea-pig (Adams, 1958) or mouse (McKenzie, 1958). The maximum level of ^{131}I in the plasma of the test animal, as measured in the assay method of Adams & Purves (1955), is found at about 3 hours after injection of extracts of mammalian pituitaries or plasma from hypothyroid animals. The sera from hyperthyroid patients produce a response that is at its maximum about 14 hours after the injection. Moreover, it has recently been found in our laboratory that the agent present in the blood of hyperthyroid patients remains for a long time in the circulation of a rat after intravascular injection, its concentration falling with a half-life of about seven-and-a-half hours, which is 30 times that of injected TSH. This thyroid-stimulating agent has been called "abnormal TSH" (Adams, 1958). The term "abnormal thyroid stimulator" is perhaps preferable, as the site of production of the material is not yet established and "TSH" suggests to some people a substance necessarily of pituitary origin. Recent work in our laboratory has shown that the abnormal thyroid stimulator is fully active in hypo-

physectomized mice, indicating that it acts directly on the thyroid. The abnormal thyroid stimulator produces its characteristic response in the rat as well as in the mouse and guinea-pig though the latter animals are much more sensitive to it, as they are to TSH.

The positive responses obtained with the sera of hyperthyroid patients in the tadpole and the chick by other investigators must also be ascribed to the abnormal thyroid stimulator rather than to TSH. In view of the fact that the abnormal thyroid stimulator stimulates the thyroid in a wide range of vertebrates, it seems almost certain that it is active in the same way in those patients in whose blood it is found and is, therefore, responsible—directly or indirectly—for the non-suppressible thyroid activity of clinical hyperthyroidism. It is remarkable, accordingly, that the amounts of abnormal thyroid stimulator found in sera correlate more closely with the degree of exophthalmos than with the degree of hyperthyroidism. In most cases of hyperthyroidism with minimal eye signs we have been able to detect the abnormal thyroid stimulator only after applying a concentration procedure. It is hardly possible to doubt that there is a direct relationship between the abnormal thyroid stimulator and the exophthalmos. The abnormal thyroid stimulator may be the cause of the exophthalmos and may be identical with the exophthalmos-producing factor found in sera by Dobyns & Wilson (1954). Alternatively it may be that the abnormal thyroid stimulator has its origin in the diseased tissues of the orbit. We did not find any abnormal thyroid stimulator in extracts of normal human orbital tissues, nor have we been able to detect it in extracts of normal human pituitary tissue. Further clarification of the obviously complex causal relationships operating in the Graves syndrome will result from the identification of the source of the abnormal thyroid stimulator.

10. Modification of the Endogenous TSH Secretion Rate

a. TSH Suppression

The secretion of TSH is controlled by the thyroid hormone level in the blood. If this be raised by the administration of an adequate dose of exogenous thyroid hormone, the secretion of TSH is completely suppressed. In the adult human subject the suppressive dose of thyroid hormone is 75–100 μg./day of L-tri-iodothyronine, or 300–400 μg./day of L-thyroxine. The equivalent amount of desiccated thyroid is about 200–250 mg./day (3–4 grains/day). These doses are only slightly more than the full therapeutic doses required in the treatment of myxoedema.

b. TSH Suppression as a Diagnostic Aid

The administration of suppressive doses of one or other form of thyroid hormone to the euthyroid subject will promptly inhibit the secretion of endogenous thyroid hormone and will eventually reduce the ^{131}I uptake to a low level. Cases of simple goitre which result from iodine deficiency may show high ^{131}I uptake values but can be differentiated from hyperthyroid cases by observing the effect of TSH suppressive treatment for one week on the results of a subsequent ^{131}I uptake test. As has been stated earlier, in hyperthyroidism the thyroid activity is not suppressed by the administration of exogenous thyroid hormone. TSH suppression tests of this kind have been described by Werner, Hamilton & Nemeth (1952), Morgans, Oldham & Trotter (1952), Greer & Smith (1954), and Werner (1956).

c. TSH Suppression as a Therapeutic Measure

The administration of desiccated thyroid to suppress TSH secretion has been advocated by Greer & Astwood (1953) for the treatment of simple goitre. It may be expected that TSH suppression will be at least as effective as the administration of iodine and, in certain cases, more effective. TSH suppression is particularly indicated in those cases of goitrous cretinism in which there is an inborn error of metabolism which prevents the thyroid from using iodine effectively to produce thyroid hormone (Stanbury & Querido, 1956).

From recent developments it appears that TSH suppression has an important place in the medical treatment of neoplastic disease of the thyroid gland. Experimental observations by Purves & Griesbach (1947) showed that thyroid neoplasms in the rat were generally under the control of the circulating TSH and were stimulated to grow by high TSH levels, but regressed and disappeared in the absence of TSH.[1] These observations led Purves and Griesbach to suggest that TSH suppression might influence the course of malignant thyroid disease in human subjects. Sturgeon, Davis, Catz, Petit & Starr (1953) reasoned that the acceleration of ^{131}I metabolism in certain human thyroid cancers by TSH indicated that these cancers were in all probability also susceptible to its growth-stimulating effect and that the suppression of endogenous TSH might, therefore, retard the growth of cancer and the occurrence of metastases. More direct evidence of the importance of endogenous TSH levels in human thyroid malignancy was provided by the frequency with which thyroid destruction by ^{131}I therapy or the surgical ablation of the thyroid preparatory to ^{131}I therapy was followed by a stimulation of the growth of a previously slow-growing neoplasm or by the appearance of a more malignant anaplastic type of growth. A most impressive collection of occurrences of this sort has been presented by Crile (1957). Balme (1954) reported the successful treatment of a single case of metastatic carcinoma of the thyroid by the administration of thyroxine. These observations warranted the trial of TSH suppression therapy as a routine measure in the management of malignant disease of the thyroid gland. Crile (1955), Maloof, Vickery & Rapp (1956) and Thomas (1958) have advocated TSH suppression therapy, and the results have been most encouraging. The beneficial effects of TSH suppression are limited to those tumours which show a well-differentiated structure, i.e., the papillary and follicular types—the more anaplastic tumours are not TSH-dependent. Crile, McNamara & Hazard (1959) report a striking reduction in the incidence of post-operative recurrence of papillary carcinoma in patients treated with desiccated thyroid after operation. Thomas (1959) reports the regression of primary tumours and of lymph-node, pulmonary and bony metastases in selected patients. These results justify the recommendations by Crile (1957) that TSH suppression should be tried on all patients with inoperable carcinomata before resorting to ^{131}I therapy and that all operable cases should be treated by TSH suppression after operation.

11. Uses of TSH Preparations

The use of TSH as a replacement therapy in pituitary failure is unnecessary. Its effects are exerted exclusively on the thyroid, and the desired effect—an increased supply of thyroid hormone to the tissues—can be obtained more conveniently,

[1] See I. Doniach, p. 99 of this number of the Bulletin.—Ed.

and controlled more precisely, by the administration of thyroid substance by mouth. This is fortunate, since any continued use of TSH is impracticable with the presently available preparations because of antibody formation. The thyroid-stimulating hormones of different mammalian species are species-specific proteins with a common biological action and act as antigens when injected into another species. Antibodies are formed that are capable of neutralizing not only the material injected but also pituitary extracts from other species and even the secretion of the treated animal's own pituitary. The subject of antihormones has been reviewed by Thompson (1941).

The response of the thyroid to a short course of injections of TSH is a valuable diagnostic aid in differentiating primary thyroid failure from thyroid inactivity secondary to pituitary failure, and is, for this purpose, more convenient than TSH assay of the blood. The ^{131}I uptake of the inactive thyroid in hypopituitarism is raised to normal by injections, given twice daily, of 12.5 i.u. TSH for 3–9 days (Querido & Stanbury, 1950; Herloff, Levy & Despopoulos, 1951). In primary hypothyroidism there is no response.

TSH injections have been used to stimulate the ^{131}I metabolism of functioning thyroid tumours (Sturgeon *et al.* 1953; Catz, Petit, Schwartz, Davis, McCammon & Starr, 1959). These authors maintained a continuous TSH suppression by thyroid medication and injected 10 i.u. TSH daily for seven days before administering either test doses or therapeutic doses of ^{131}I.

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NATURALLY OCCURRING GOITROGENS

F. W. CLEMENTS M.D.

Institute of Child Health
University of Sydney, Australia

1 Historical review
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1. Historical Review

a. From Recognition to Chemical Isolation

It is over 30 years since Chesney and his co-workers (Chesney, Clawson & Webster, 1928; Webster, Clawson & Chesney, 1928; Webster & Chesney, 1930) announced the production of large goitres in rabbits being used to study experimental syphilis. Consideration of the diet in these experiments and subsequent tests by other workers (for full reviews of these experiments see Greer, 1950, 1956) proved that the cabbage, which was the main item in the diets in these experiments, was goitre-producing. While some later experiments confirmed this, others did not; also it became clear that cabbage grown in different parts of the world and in different seasons varied widely in goitrogenic properties. Nevertheless, by the early 1930's the fact had been established that some foods were goitrogenic. Hercus & Purves (1936) in New Zealand, after failing to produce goitre in experimental animals with a diet of cabbage, demonstrated that the seeds of cabbage, rape and mustard contained a goitrogenic principle. It was noted that, whereas additional iodine prevented "cabbage goitre", it only partly inhibited the effects of the active substance in the seeds.

The pioneer work of the MacKenzies (MacKenzie, MacKenzie & McCollum, 1941), in which they showed that sulphaguanidine produced goitre in rats, the parallel studies by another Boston group (Richter & Clisby, 1941), demonstrating the goitrogenic properties of phenylthiourea, and the series of investigations in New Zealand (Kennedy, 1942) which confirmed the goitrogenic properties of the seeds of a number of *Brassica*, paved the way for the isolation by Astwood and his co-workers (Astwood, Greer & Ettlinger, 1949) of an active goitrogenic compound, L-5-vinyl-2-thio-oxazolidone, from the seeds of a number of *Brassica* and from the roots of swedes and turnips. This was slightly more goitrogenic for man than propylthiouracil and had about one-fifth the potency of the same drug for the rat. Tests with ^{131}I indicated that action on the thyroid gland was similar to that of the antithyroid drugs of the aminobenzene and thioamide series; it blocked the organic binding of iodine.

Greer (1956) proved that vinyl thio-oxazolidone (goitrin) was present in the seeds and roots as an inactive precursor which required enzymic action for conversion to the active form. Cooking destroyed the enzyme and so the goitrogenic properties. Greer (1957) found that the progoitrin content of the seeds of *Brassica* varied from batch to batch and from season to season even for the same variety. It is noteworthy that until recently progoitrin had not been demonstrated in cabbage or the other species of *Brassica* used in the early experiments to produce cabbage goitre (Greer, 1956). However, it has now been shown to be present at a concentration of one part in eight million in cabbage, "a quantity which is probably negligible in practical nutrition" (Altamura, Long & Hasselstrom, 1959). Some other substance must have been responsible for the results obtained by Chesney and his co-workers.

b. Goitre in Domestic Animals

Goitre occurs in many types of livestock and in some instances this has been shown to be associated with a particular diet. Care (1954) in New Zealand showed that linseed meal when fed to pregnant ewes produced large goitres in the new-born lambs, which he proved to be due to a thiocyanate derived from the detoxication of the cyanide-producing glucoside, linamarin, present in the seeds. These effects could be eliminated by adding iodide to the diet; this is in keeping with the accepted action by the thiocyanates upon the thyroid (Astwood, 1943). Sinclair & Andrews (1954), investigating a severe outbreak of goitre, with heavy neonatal mortality, in lambs from kale-fed ewes in New Zealand, showed that the effects could be controlled with additional iodide. Another report from New Zealand confirms this (Wright, 1958), while there is also a suggestion (New Zealand Department of Agriculture, 1957) that some, at least, of the goitrogenic action of kale may be due to a thyroid-blocking agent, like thiouracil. Whittem, investigating an outbreak of goitre in new-born lambs in Tasmania in 1956, concluded that this was due to the consumption by the pregnant ewes in the last month of pregnancy of particularly lush growth of the common crow's foot (*Erodium cicutarium*) and the long stork's bill (*Erodium botrys*). The overgrowth of these plants was associated with a warm humid autumn and short warm winter (Whittem, personal communication, 1956). The causal agent was not isolated.

c. Goitrogens in Edible Nuts

Moudgal and his co-workers (Moudgal, Srinivasan & Sarma, 1957; Moudgal, Raghupathy & Sarma, 1958) at the University of Madras, as the result of a number of studies on glycosides obtained from the red outer skin-covering of some edible nuts, including peanuts, concluded that the goitrogenic action of these nuts is probably due to a double action—the interference with the uptake of inorganic iodine and the blockage of the organic binding of iodine by tyrosine. These actions were only partially inhibited by supplemental iodine.

d. Summary of Evidence for Naturally Occurring Goitrogens

The results of these various studies have demonstrated that there are present in plants and their seeds a number of substances which are actually or potentially goitrogenic for man, his domesticated animals and some experimental animals. It would appear that there are at least two different kinds of compounds. One produces goitre by preventing the uptake of iodine by the thyroid gland; the thiocyanates are an example

of this type of substance. The other group produces goitre by blocking the organic binding of iodine; these are the substances for which Astwood (1945) suggested the name "anti-thyroid compounds"; goitrin or L-5-vinyl-2-thio-oxazolidone belongs to this group.

2. Effects of Naturally Occurring Goitrogens on Man

a. Food-Stuffs Containing Thiocyanates

The thiocyanate may not be present in the plant as the free ion, but as a glycoside which is converted in the animal body to a thiocyanate. Since the action of thiocyanates on the thyroid can be controlled by supplemental iodine, food-stuffs potentially rich in thiocyanates may not produce thyroid enlargement in persons on a diet with a content rich in iodine. Ground-nuts and the members of the cabbage family are eaten by man, but the other plants which produce goitre by releasing thiocyanate are used as food only for livestock.

So far as is known, thyroid enlargement has not been produced in man by the consumption of these foods through the action of thiocyanate present or derived from precursors. A possible explanation for this is to be found in the low intake of thiocyanate from these sources in relation to the dosage which has been found necessary to produce thyroid enlargement in man. Michajlovskij & Langer (1958) have analysed a variety of plants, including a number of species of *Brassica*, for thiocyanate content. Values ranging from 2.2 mg./100 g. for kohlrabi to 10.2 mg./100 g. for cauliflower were obtained. These workers further showed that rises occur in blood thiocyanate levels after the ingestion of comparatively large amounts of cabbage and other species of *Brassica* (Langer & Michajlovskij, 1958). However, the range of values obtained, namely 0.5–2.0 mg./100 ml., was well below the range found in patients with hypertension treated with thiocyanates who developed thyroid enlargement. Definite enlargement of the thyroid occurred in a significant number of patients given this form of therapy when the thiocyanate blood level was maintained between 8 mg./100 ml. and 10 mg./100 ml. for prolonged periods (Wald, Lindberg & Barker, 1939). To obtain these levels a dose of 1.0 g. daily of potassium thiocyanate was frequently necessary. This would correspond to a regular daily consumption of about 10 kg. cauliflower or kale. Furthermore it was the experience of physicians in the 1930's, when this form of therapy was fashionable, that the dosage of thiocyanate had to be progressively increased to maintain blood levels necessary to affect hypertension. Thus it would seem that, in man, the thiocyanate blood levels necessary to produce thyroid enlargement are at least four times higher than the highest figures recorded after the ingestion of foods rich in thiocyanates. It is, therefore, reasonable to assume that, while the ingestion of staple feeding-stuffs rich in thiocyanates can produce a goitre in experimental and domestic animals because of quantities consumed, it is unlikely, except in the most exceptional circumstances, that a diet of foods rich in thiocyanates will produce goitre in man for the reverse reason—he does not consume enough of the food-stuff.

b. Food-Stuffs Containing Thyroid-Blocking Substances

Three thio-oxazolidones have been isolated from plants:

i. 5:5-Dimethyl-2-thio-oxazolidone was originally isolated from *Conringia orientalis* (hare's-ear mustard) which infests the wheat-fields of western Canada (Hopkins, 1938; Kjaer, Gmelin & Jensen, 1956). It has also been found in the seeds of *Cochlearia* species, notably *Cochlearia officinalis*. This substance was found to be goitrogenic (Astwood, Bissell & Hughes, 1945).

ii. L-5-Vinyl-2-thio-oxazolidone has been isolated from the seeds of many species of *Brassica* and the roots of swedes and turnips. Its goitrogenic activity has been repeatedly demonstrated (Astwood *et al.* 1949). It requires enzymic action for its activation and since these foods are usually cooked before being eaten any potential goitrogenic action would be destroyed. For this reason the discovery of this substance in human food has been considered to be largely of academic interest.

iii. 5-Phenyl-2-thio-oxazolidone has been derived from the seeds of *Barbarea vulgaris* (winter-cress) and also from the seeds, leaves, stems and roots of *Reseda luteola* (dyer's weed, weld) by enzymic hydrolysis (Kjaer & Gmelin, 1957, 1958). *Barbarea* is one of the Cruciferae, but *Reseda* belongs to the closely related Resedaceae. So far as is known, this compound has not been tested for goitrogenic properties, but both 3-phenyl-2-thio-oxazolidone and 4-phenyl-2-thio-oxazolidone have been tested and found to have low activity (Astwood *et al.* 1945).

These studies suggest that the direct ingestion of food-stuffs with high goitrogenic activity by man is unlikely. However, it is possible for goitrogenic substances to be acquired by human beings in another way, namely in milk from cows grazing on pastures or forage crops which contain either goitrogens or progoitrogens. The conditions existing in the rumen would not prevent the enzymes in the plants from converting a progoitrogenic substance to the active form. For this type of goitrogen to produce goitre in man and his children it is necessary to demonstrate that: (i) pastures and forage crops contain these goitrogens or their precursors, and (ii) milk from cows feeding on these crops is goitrogenic.

i. Goitrogens in Pastures and Crops

As indicated above, one substance has been isolated from hare's-ear mustard by Hopkins in Canada. This plant is reported to invade the wheat-fields and could presumably contaminate pastures. The habitat of many of the cruciferous weeds, including hare's-ear mustard and Queensland wild turnip weed (see below), is such that the plant at all stages of development, including the seeds, could be eaten by grazing cows.

An examination of the thioglucosides and their aglucones occurring in weeds contaminating pastures in goitrous areas of Tasmania and Queensland is proceeding at the School of Biochemistry, University of Melbourne, and γ-methylsulphonyl propyl*iso*thiocyanate has been isolated from the fruit and leaves of the Queensland wild turnip weed (*Rapistrum rugosum*) and shown to reduce the uptake of ^{131}I by the thyroid gland of the rat (dose, 5 mg.). The yield of pure aglucone (1.2 g./kg. dry fruit and 0.4 g./kg. fresh leaves) would indicate a potentially high consumption of the substance by a grazing cow during a 24-hour period, since many pastures in southern Queensland are heavily infested with this weed (Bachelard & Trikojus, personal communication, 1959).

ii. Goitrogenic Properties of Milk

The evidence for the presence of a goitrogen in milk is conflicting. Clements carried out a series of tests on human

volunteers with samples of milk obtained from cows fed a number of different types of rations (Clements & Wishart, 1956; Clements, 1958). The test was the standard one employed to measure the antithyroid potency of substances (Stanley & Astwood, 1947; MacGregor & Miller, 1953). The principle of the test is that after the administration of a tracer dose of radioactive iodine, the rate of uptake of ^{131}I by the thyroid, plotted against the square root of time, follows a straight line for 10–12 hours. A dose of an antithyroid substance given after the form of the uptake curve has been established changes the direction of the curve. Within limits, the extent of the deflection of the curve from the straight line is a measure of the potency of the substance.

Figure 1 (curves A–F) shows the results obtained from tests on samples of milk from cows fed on:

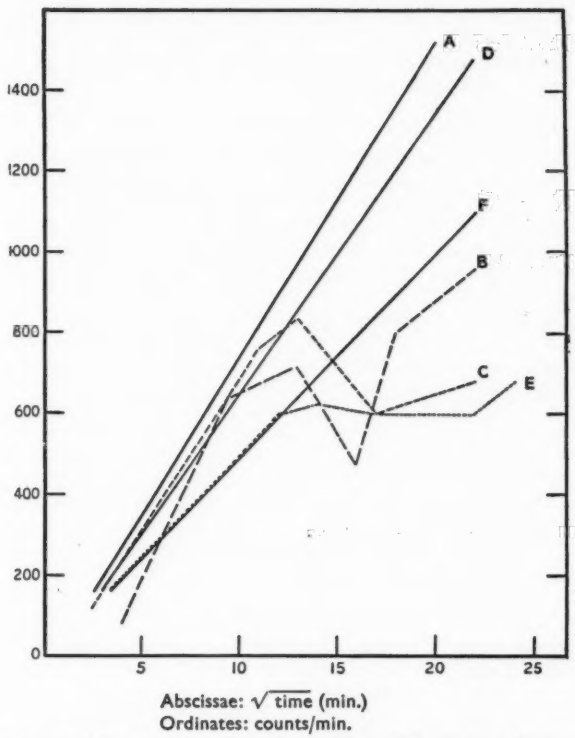
- A: mixed grass pastures uncontaminated with weeds;
- B: a grass pasture with about 25% contamination by the cruciferous weeds, lesser swine's cress, pepper-cress and shepherd's purse;
- C: chou moellier (kale), forming about 30% of food requirements; the milk was obtained from southern Tasmania;
- D: chou moellier (kale), forming about 30% of food requirements; the milk was produced on experimental farms of the University of Sydney;
- E: pastures heavily contaminated with Queensland turnip weed;
- F: pastures plus about 20% of food requirements in the form of turnips.

Curve C (fig. 1) is an example of the curves obtained from a number of samples of this type of milk collected from Tasmania. However, about one-third of the samples forwarded from farmers who claimed to have fed such rations failed to affect the uptake of ^{131}I. It will also be noted that milk from cows at the University farms in New South Wales fed on approximately the same ration did not affect the uptake of the radioactive iodine (curve D). This test was made on one of the adults who had participated in the tests on the Tasmanian milk.

The results obtained by Clements with milk from cows fed on pastures contaminated with the cruciferous weeds, lesser swine's cress (*Coronopus didymus*), pepper-cress (*Lepidium ruderale*) and shepherd's purse (*Capsella bursa pastoris*) are of particular interest when compared with the results obtained by Greene, Farran & Glascock (1958) in England. In attempting to reproduce Clements' work they tested milk from cows fed chou moellier, and milk from cows fed hay and pastures lightly contaminated with cruciferous weeds of species not stated. They found the latter to be more goitrogenic than milk from cows fed chou moellier, although the interference with thyroid metabolism was not as marked as that obtained by Clements.

Van der Veen & Hart (1955) in Holland fed North Holland Blue cocks, twelve weeks old, with milk from cows which had been fed on turnips, and found no evidence of goitrogenic activity despite the fact that the turnips contained about 10 mg. L-5-vinyl-2-thio-oxazolidone/kg. Kreula & Kiesvaara (1959) working in Helsinki, having developed a method for the microdetermination of L-5-vinyl-2-thio-oxazolidone in milk, estimated the amount of this substance transferred to the milk when cows were fed green turnip, rape or kale in which

FIG. 1. Curves of Thyroidal Uptake of Radioiodine



Abscissae: $\sqrt{\text{time}}$ (min.)
Ordinates: counts/min.

Curves A–F show results obtained with milk from cows fed on various diets. (See text section 2b.)

Curves B, C and E show some interference with uptake of ^{131}I.

The subjects in these tests consumed at least one-and-a-half pints of milk in about one hour. The milk was obtained from cows fed a variety of fodders, as indicated in the text.

the amount of vinyl thio-oxazolidone had been determined. They found that about 0.05% was transferred to the milk, and that the percentage transferred was the same when crystalline vinyl thio-oxazolidone instead of *Brassica* was given to the cow. They rightly concluded that this quantity would not be sufficient to produce a goitre. These workers also noted that this substance disappears rapidly from milk if it is not heated immediately after milking. They suggest that if milk from cows fed *Brassica* is goitrogenic it is owing to other substances, which they are now seeking (Virtanen, personal communication, 1959).

Clements & Wishart (1956) have demonstrated that ethanolic extracts of freeze-dried skim-milk from cows fed chou moellier and grazing on pastures heavily contaminated with Queensland turnip weed (*Rapistrum rugosum*) prevented the uptake of ^{131}I in rats in the same manner as Neo-Mercazole.

The results of these various experiments are conflicting. At this stage the only conclusion to be drawn is that goitrogenic substances have not yet been isolated from milk, although there is strong evidence from tests with whole milk, skim-milk and extracts of skim-milk that milk from cows feeding on some crops or pastures interferes with the uptake of ^{131}I by the

thyroid, whereas milk from cows feeding on different pastures has no such effect. Laboratory tests in Finland would indicate that, if a goitrogen is present in milk from cows feeding on crops known to contain vinyl thio-oxazolidone or some other similar compound, the goitrogenic activity of the milk is not due to vinyl thio-oxazolidone.

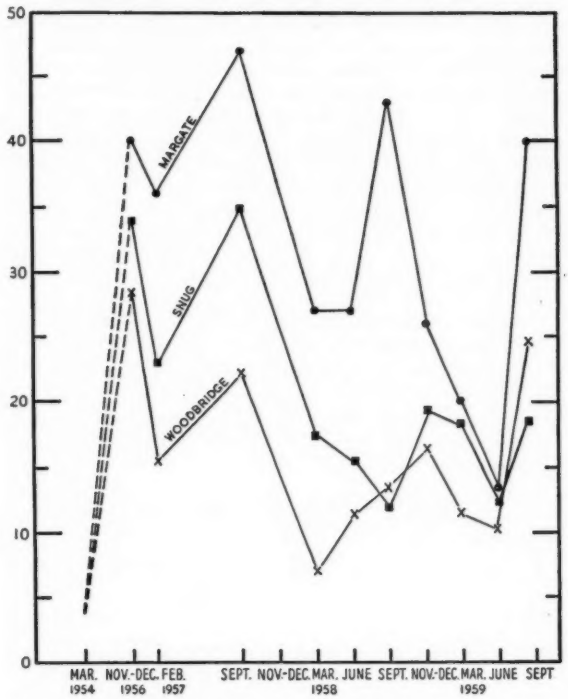
iii. Epidemiological Studies of Children with Goitre

There is no question that in many parts of the world where endemic goitre occurs it is due to an insufficient intake of iodine: this has been conclusively demonstrated by the administration to the susceptible population of supplemental iodine (Marine & Kimball, 1921; Scrimshaw, Cabezas, Castillo & Méndez, 1953; Stacpoole, 1953; Clements, 1958). That other factors may be responsible for the occurrence of endemic goitre has been suggested by a number of writers, and has been emphasized recently by Scrimshaw (1958). For many years Greenwald has contested the iodine deficiency theory, advancing in its place the possibility of an infective agent spread by carriers entering what would seem to be—judging from the scanty records in each instance—an unaffected area (Greenwald, 1945, 1952, 1958). That there may be another explanation for the sequence of events assembled by Greenwald is suggested later in this paper. Some goitre surveys, in which an attempt is made to correlate the incidence of goitre with the intake of iodides, challenge the iodine deficiency theory, as has been recently pointed out by the *Scottish Medical Journal* (1959).

Studies in Tasmania and some other parts of Australia lend support to the idea that iodine deficiency is not the only cause of endemic goitre (Clements & Wishart, 1956). Gibson, Howeler & Clements (1960) have recently completed an epidemiological study of a group of children attending three schools in southern Tasmania, about 30% of whom have shown a seasonal enlargement of the thyroid for at least the past three years—the period of intensive study. The peaks of the epidemics occurred in the late spring and early summer, with a recession in the winter. The same children appear to be affected each year. In the same community approximately 18% of boys and 25% of girls had a normal thyroid over the period of the study, and 29% of boys and 22% of girls had a constantly enlarged thyroid. All these children had been receiving regular prophylactic doses of potassium iodide since they commenced at school, and certainly for the past three years. At one school the dose had been 10 mg. once weekly, at the other two schools the dose had been 10 mg. twice weekly (see fig. 2). Despite these dosages the seasonal epidemics continue to occur. Following the most recent survey (September 1959), Dr Heather Gibson, who has regularly made these assessments, remarked that the current epidemic seemed "fantastic" (see fig. 2)—particularly at Margate School, Tasmania. In the epidemiological study no differences were found in the physical environment, economic status, way of life, dietary pattern, milk consumption, and sources of milk of the children with a seasonal enlargement, no enlargement or a constant enlargement. It is noteworthy that in many families there were children with each of these types of thyroid.

The seasonal increase in size appears to coincide each year with the spring flush of pastures and weeds; at this time of the year there is an abundant growth of a number of weeds, but the dominant ones appear to be wild radish (*Raphanus raphanistrum*) and wild turnip (*Brassica campestris*). The coincidence of goitre enlargement with seasonal flush was emphasized in 1958, when the season was delayed about one month in the most southern part of the area studied. Children from this district attend Woodbridge Area School, Tasmania. In previous years the peak of seasonal increase had been the same at each school, namely in September. In 1958 the peak occurred at the most northern school in September, and at Woodbridge and Snug in December.

FIG. 2. Percentage Incidence of Visible Goitre in Boys attending Three Tasmanian Schools



Abscissae: date

Ordinates: incidence of visible goitre (%)

The curves show three seasonal epidemics with the peaks in the spring. The children at the Margate and Woodbridge schools have had 10 mg. potassium iodide twice weekly, and those at the Snug school 10 mg. potassium iodide once weekly for at least three years. Similar curves were obtained for girls.

An important feature of this type of thyroid enlargement is that boys and girls are affected in about the same numbers, in contrast to iodine deficiency goitre when the ratio of girls to boys affected is about 3:1. The curves for boys only are given in fig. 2; those for girls almost parallel these. Curves for both will be published later (Gibson *et al.* 1960). The peak of the seasonal enlargement occurred in children 7–10 years of age; the variation becomes less as the children's ages advance.

Gibson *et al.* (1960) have postulated that milk, which is consumed in larger amounts by young children, contains a goitrogenic substance to which some children are susceptible, but others not. The susceptible children show the changes each year and these are in no way controlled by relatively large prophylactic doses of iodide. These authors suggest that the

goitrogen is obtained from the cruciferous weeds. Trikojus's laboratory at the University of Melbourne is investigating these plants, but a specific goitrogenic or progoitrogenic substance has not yet been isolated.

In a survey of the distribution of goitre in southern Queensland, Clements (1958) has shown that goitre occurs in the valleys, where the pastures, on which cows supplying the homes with milk are grazed, are heavily contaminated with Queensland turnip weed (*Rapistrum rugosum*), whereas on the ridges, where this weed does not grow, the children are free from goitre.

iv. Goitre and the Settlement of New Lands

The usual procedure when new lands are settled is for the cows and stock to be first grazed on the indigenous grasses. At the same time land is cultivated for crops; later, when this land is allowed to lie fallow, it quickly becomes infested with all varieties of weeds which flourish more in fallowed cultivated land than in natural pasture. As farming methods advance, improved pastures are introduced, particularly for milk production, and once a tight sward is established weeds are eliminated.

This is the sequence of events which has happened and is happening in many parts of Australia, and undoubtedly in other parts of the world, and could account for the sudden appearance and then gradual disappearance of goitre under conditions in which the theory of iodine deficiency as the cause of goitre in these particular areas cannot be supported.

3. Summary

The evidence for the existence of naturally occurring goitrogens is firm: thiocyanates and thiouracil-like substances occur in plants eaten by domestic animals and, to a much lesser extent, in foods eaten by man. It seems that a thiocyanate could be responsible for a significant amount of goitre in lambs when the ewes are fed certain plants. Experiments so far conducted have excluded the possibility that a thyroid-blocking substance may also be responsible.

It is highly unlikely that plant foods rich in thiocyanates are eaten in sufficient quantities to produce goitre in man. At least one thyroid-blocking agent occurs in foods eaten by man, but since these are eaten cooked, and cooking prevents the formation of the active form, it is highly unlikely that this substance, obtained directly from the plants, is a factor. The possibility that goitrogens might be acquired from milk has been explored. The evidence is inconclusive and still of an indirect nature. One possible goitrogen, γ-methylsulphonyl propyl-*iso*thiocyanate, has been isolated from a cruciferous weed which is known to be eaten by cows in a district where there is a high incidence of goitre in human beings. A goitrogenic substance has not yet been isolated from the milk, although the investigations are proceeding.

Epidemiological studies of children in goitrous regions, some of whom have been given prophylactic iodine for a number of years, strongly suggest that a goitrogen probably present in the milk is responsible for much of the thyroid enlargement, especially for the seasonal epidemics of goitre.

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THYROID HORMONES AND PLASMA LIPIDS

G. S. BOYD Ph.D.

Department of Biochemistry
University of Edinburgh

M. F. OLIVER M.D. F.R.C.P.E.

Department of Cardiology
The Royal Infirmary, Edinburgh

1 The plasma lipids
2 Thyroxine analogues
3 Studies in animals
4 Studies in man
a Hypothyroid patients
b Euthyroid patients
5 Discussion
References

The clinical appraisal of thyroid function has been established on a firm basis for many years and, with the introduction of methods for determination of the plasma lipids, it soon became apparent that there is an approximate inverse relationship between thyroid activity and plasma cholesterol levels. In man, plasma cholesterol is usually elevated in spontaneous and induced hypothyroidism (Mason, Hunt & Hurxthal, 1930; Hurxthal, 1934; Gildea, Man & Peters, 1939). In spontaneous and induced hyperthyroidism, the plasma cholesterol is frequently below normal (Epstein & Lande, 1922; Mason *et al.* 1930). A precise relationship between changes in plasma cholesterol and changes in the basal metabolic rate (BMR) is not always present (McGee, 1935). For example, reduction of the BMR can occur in euthyroid subjects without associated hypercholesterolaemia (Mason *et al.* 1930); and substances such as dinitro-*o*-cresol can elevate the BMR without reducing the high plasma cholesterol level of myxoedema (Dodds & Robertson, 1933). Thus, measurements of plasma cholesterol and of the BMR do not always give the same assessment of thyroid status.

1. The Plasma Lipids

Almost all the lipids in plasma are bound to two major protein fractions, namely the α- and β-globulins, and the plasma lipids circulate as α- and β-lipoproteins. The basic unit of each lipoprotein is a macromolecular complex of triglycerides, phospholipids, cholesterol (free and esterified) and protein. In health, the triglyceride content of plasma varies greatly and depends largely upon the phase of intestinal absorption of fat, while the phospholipid and cholesterol contents are relatively constant. The α- and β-lipoproteins are heterogeneous and can be fractionated by various physical means, such as electrophoresis or centrifugation, into subgroups of varying molecular weight. Since these various plasma lipids are interdependent variables, alteration of one frequently results in changes in the others; for example, in man a rise in the plasma cholesterol level is associated with an increased cholesterol:phospholipid ratio (C:P ratio), a decreased ratio of α-lipoprotein to β-lipoprotein and a rise in certain β-lipoprotein sub-fractions. There are various methods of determining and describing the plasma lipoproteins but, since there are different opinions as to the effects of thyroactive compounds on the plasma lipoprotein pattern (Malmros & Swahn, 1953; Boyd & Oliver, 1957; Strisower, 1959), we will describe the effects of the thyroid hormone on the plasma lipids chiefly in terms of the plasma cholesterol.

Elevation of the plasma cholesterol concentration is present in many patients with coronary heart disease. Although there is no definite evidence of a relationship of cause and effect, it is argued, for reasons which are beyond the scope of this review, that reduction of hypercholesterolaemia is desirable in patients with coronary heart disease and in apparently healthy individuals.[1]

2. Thyroxine Analogues

The administration of thyroid hormones in order to reduce high plasma cholesterol levels in patients with coronary heart disease is greatly limited by their stimulation of oxygen requirements. The myocardial blood supply of such patients is frequently sufficient to meet only limited demands, and their coronary arteries are so diseased that any great increase in myocardial oxygen requirements can seldom be met. Thus, an increase in myocardial metabolism may result in a discrepancy between demand and supply, and myocardial ischaemia and angina may result.

Thyroxine has many physiological effects, including a decrease in tissue cholesterol content and stimulation of tissue oxygen consumption. If these effects are mediated through independent pathways, it may be possible to influence one and not the other. Thus, alteration in the structure of thyroxine might result in a thyroactive compound more active in lowering plasma cholesterol than in increasing tissue oxygen demands. The thyroxine molecule is a halogenated diphenyl ether attached to an alanine side-chain containing an asymmetric centre. This molecule containing many reactive groups can be modified by enzymes capable of de-iodination, inversion of the optical centre, deamination and decarboxylation of the amino-acid residue. Theoretically these changes would produce many metabolites. Certain of these "theoretical metabolites" have been prepared synthetically and assayed for their metabolic effects in animals and man (Lerman, Harington & Means, 1952; Roche, Michel, Wolf & Etling, 1954; Michel & Pitt-Rivers, 1957; Mussett & Pitt-Rivers, 1957). The discussion of these is beyond the scope of this paper, but the effect of certain thyroxine analogues on oxygen consumption, myocardial metabolism and plasma cholesterol levels will be reviewed.

3. Studies in Animals

Our principal interest has been the effect of thyroxine analogues on the plasma lipids, but certain other assays were necessary to enable us to evaluate any additional physiological effects of doses which lower the plasma lipids. The animals used in these studies were inbred male albino rats of the Wistar strain. The following experiments were undertaken.

[1] See Bronte-Stewart, *Brit. med. Bull.* 1958, 14, 243.—Ed.

TABLE I. Dose Range of Thyroactive Compounds Required to Elicit Responses Shown by Column Headings

(Values are given in mg./kg. of diet.)

No.	Thyroactive compound*	50% increase in oxygen consumption rate	50% increase in heart rate	50% increase in heart weight	50% depression of serum cholesterol	Complete suppression of goitre
1	3:5:3′:5′-Tetraiodo-L-thyronine (L-thyroxine)	2–5	2–5	2–5	1–3	0.3–0.6
2	3:5:3′:5′-Tetraiodo-D-thyronine (D-thyroxine)	100–200	150–300	200–400	7–10	2–4
3	3:5:3′:5′-Tetraiodothyroacetic acid (TETRAC)	50–200	1,000	—	10–100	1–10
4	3:5:3′:5′-Tetraiodothyroformic acid	1,200	500–1,500	1,000–2,000	100–250	100–200
5	3:5:3′:5′-Tetraiodothyro-amine	—	1,000–3,000	1,000	150–750	—
6	3:5:3′-Tri-iodo-L-thyronine	0.2–0.6	0.2–0.6	0.2–0.6	0.5–1.0	0.01–0.10
7	3:5:3′-Tri-iodo-D-thyronine	5–20	10–30	5–15	0.7–2.0	0.5–1.0
8	3:5:3′-Tri-iodothyroacetic acid (TRIAC)	5–30	10–20	5–10	0.5–5.0	0.5–3.0
9	3:5:3′-Tri-iodothyroformic acid	—	—	—	50–100	—
10	3:5-Di-iodo-L-thyronine	20–40	30–60	20–50	20–70	1–2.5
11	3:5-Di-iodo-D-thyronine	300–600	500–1,000	700–1,100	50–150	10–35
12	3:5-Di-iodothyroacetic acid	300–600	400–900	500–1,000	50–100	10–50
13	3:5-Di-iodothyroformic acid	2,500 and upwards	2,500 and upwards	2,500 and upwards	1,000 and upwards	50–100

* Nos. 3, 4 and 5 refer to analogues of thyroxine in which the alanine side-chain is replaced by acetic acid, formic acid, or an ethylamine group; 8 and 9 are the corresponding analogues of tri-iodothyronine; 12 and 13 are the corresponding analogues of di-iodothyronine.

i. *Oxygen consumption.* The rate of oxygen consumption was determined (in the morning) in the fasting state at 25° C. by the technique of Maclagan & Sheehan (1950) as modified by Tomich & Woollett (1953). Each thyroxine analogue was administered at three dose-levels, and the rate of oxygen consumption measured every three days over a 14-day period: six animals were used to assess each dose-level and therefore 18 animals were used for each analogue. The effects of certain analogues in these assays are shown in Table I.

ii. *Heart rate.* The same groups of animals were used for this investigation as for the oxygen consumption assay. The animals were very lightly anaesthetized with ether. Their heart rates were recorded by a photographic plate electrocardiogram with fast-running paper, using subcutaneous electrodes. These results are also shown in Table I.

iii. *Heart weight.* The same groups of animals were also used in this investigation. After 14 days' administration of the thyroxine analogue, the animals were killed and weighed, the hearts removed at once, washed and fixed in 10% formalin. The following day, the large vessels were removed and the hearts prepared in a uniform way, blotted and weighed. The results were expressed as the ratio of heart weight to body-weight. The effect of each thyroxine analogue on this ratio is shown in Table I. The cardiac hypertrophy induced by thyroxine analogues was accompanied in certain instances by distinctive histological changes to be described elsewhere by Boyd and Montgomery.

iv. *Serum cholesterol levels.* The animals employed in this study were rats maintained on a stock semisynthetic diet (Cuthbertson, Elcoate, Ireland, Mills & Shearley, 1959). This diet was supplemented with 10% olive-oil and 1% cholesterol, and the thyroxine analogue under study. The serum cholesterol of the animals was determined after 14 days on this diet and compared with that of two control groups—one without the thyroxine analogue and the other without the olive-oil and cholesterol supplement. The dose-levels of each analogue which were required to produce depression of serum cholesterol are shown in Table I.

v. *Antigoitrogenic assay.* The technique employed was identical with that described by Gross & Pitt-Rivers (1953), except that both thiouracil and the thyroactive compound under test were incorporated into the diet. The period of the test was 14 days, and each assay consisted of at least three dose-levels with a minimum of six animals per dose. The standard used for each analogue was L-thyroxine. The results of these antigoitrogenic assays are shown in Table I.

It is concluded that a given thyroxine analogue produced closely parallel effects on heart rate, heart weight and rate of oxygen consumption. The heart rate seems to be directly related to both the heart weight and the rate of oxygen consumption of the whole body, but the depression of the serum cholesterol is less closely related to these cardiac effects. Thus, the ratio of the effect on the serum cholesterol to the effect on the heart rate can be used as a practical guide to the possible selection of compounds for clinical trials, on the assumption that it is desirable to obtain a compound with a greater effect on serum cholesterol than on heart rate. The least favourable compounds in respect of this ratio (Table I) appear to be the same compounds that produce angina most readily in man—e.g., 3:5:3′-tri-iodo-L-thyronine and 3:5:3′-tri-iodothyroacetic acid (TRIAC)[2], whereas analogues which affect the ratio more favourably include D-thyroxine, 3:5:3′-tri-iodo-D-thyronine, 3:5-di-iodo-D-thyronine and 3:5:3′:5′-tetraiodothyroformic acid[2].

The results of the effect of the antigoitrogenic assay are included in Table I, since it was hoped to compare the effect of thyroxine analogues on serum cholesterol with their effect on pituitary suppression. It was thought that this animal

[2] See footnote to Table I.

assay might give information concerning the mechanism of escape from cholesterol depression observed in man, after the continued administration of certain analogues (see section 4b). This did not prove to be the case, and the details of the action of these thyroxine analogues in this and other assays will be published shortly by us.

4. Studies in Man

a. Hypothyroid Patients

Investigations of the effect of thyroxine analogues on the plasma lipids in hypothyroid patients were conducted in the following way. After a 5–7-day control period, the thyroxine analogue under study was administered for 14 days and suitable doses of L-thyroxine were substituted thereafter. Before and during the experimental period when the thyroxine analogue was under study, the fluid balance, plasma cholesterol level and the BMR were recorded repeatedly.

Usually the first effect of administration of these compounds to hypothyroid patients was a diuresis, and within a day or two a fall occurred in plasma cholesterol. Elevation of the BMR did not always occur simultaneously, and it was often necessary to increase the dose to raise the BMR. Reduction of the plasma cholesterol without elevation of the BMR was seen with all these compounds, but this dissociation may depend partly on the duration of administration and may be lost with time. Some of the thyroactive compounds shown in Table II have a very rapid action, and recently Ibbertson, Fraser & Alldis (1959) have emphasized that both 3:5:3′-tri-iodo-L-thyronine and TRIAC produce discernible effects after four hours, and most of the physiological effects of increased thyroid function after 12 hours.

We have administered nine iodinated thyroxine analogues to hypothyroid patients, and the minimal dose which reduced the plasma cholesterol is shown for each analogue in Table II, which also incorporates the results of similar studies by other investigators. There have, of course, been numerous reports of the effects of L-thyroxine—including those of Lyon (1927); Pitt-Rivers & Lerman (1948); Hart & Maclagan (1950)—and 3:5:3′-tri-iodo-L-thyronine (Gross, Pitt-Rivers & Trotter, 1952; Asper, Selenkow & Plamondon, 1953) in myxoedema, but in these investigations the dose of the thyroactive compound was much greater than the smallest dose necessary to lower serum cholesterol.

b. Euthyroid Patients

The effects of certain thyroxine analogues on the plasma lipids were studied in euthyroid male out-patients with hypercholesterolaemia and clinical features of coronary heart disease. The objective of these studies was twofold: firstly, to determine the threshold dose of each thyroxine analogue that would lower the plasma cholesterol and, secondly, to determine the average dose that could safely be given to a majority of patients without causing angina. Studies were made only after three months had elapsed since myocardial infarction. Estimations of plasma lipids were performed on not less than three occasions during a control period of six or more weeks before treatment. The plasma lipids were estimated and the patient's weight was recorded at frequent regular intervals throughout and after the test period. The BMR and an electrocardiogram were always recorded at the beginning and at the end of each experimental period, and additional records obtained when required. Each compound was administered to groups of 12 or more men.

TABLE II. Approximate Minimal Daily Dose of Thyroid Compound Required to Reduce Plasma Cholesterol in Hypothyroid Patients

Thyroactive compound	Approximate minimal daily dose	Additional references
3:5:3′:5′-Tetraiodo-L-thyronine (L-thyroxine)	100 μg.†	Pitt-Rivers & Lerman, 1948; Asper, Selenkow & Plamondon, 1953; Malmros & Swahn, 1953; McGavack & Reckendorf, 1956
3:5:3′:5′-Tetraiodo-D-thyronine (D-thyroxine)	5 mg.†	Greene & Farran, 1958; Starr, 1958
3:5:3′:5′-Tetraiodothyroacetic acid (TETRAC)	2–4 mg.	Goolden, 1956
3:5:3′:5′-Tetraiodothyroformic acid	100 mg.†	
3:5:3′:5′-Tetraiodothyro-amine	10–15 mg.†	
3:5:3′-Tri-iodo-L-thyronine	10–20 μg.†	Gross, Pitt-Rivers & Trotter, 1952; Asper *et al.* 1953
3:5:3′-Tri-iodo-D-thyronine	100 μg.†	
3:5:3′-Tri-iodothyroacetic acid (TRIAC)	0.5–1.5 mg.	Lerman & Pitt-Rivers, 1955, 1956; Trotter, 1955, 1956; Oliver & Boyd, 1956; Boyd & Oliver, 1957; Mackay, Goble & Sparkes, 1957
3:5:3′-Tri-iodothyropropionic acid*	500 μg.	Rawson, Money, Kroc, Kumaoka, Benua & Leeper, 1959
3:5-Di-iodo-L-thyronine	3–5 mg.†	
3:5-Di-iodo-D-thyronine	50–75 mg.†	
3:5-Di-iodothyroacetic acid	50–75 mg.†	

* The propionic acid analogue of tri-iodothyronine (see footnote to Table I).

† The evidence for selection of the dose shown in this table will be published shortly by us.

It was generally found that the safe dose was about twice the threshold dose, yielding a narrow therapeutic margin. The threshold dose for each analogue is shown in Table III, which includes observations of other investigators. In our experience, it is usually possible to produce depression of the serum cholesterol without elevating the BMR. However, when the dose is continued at a constant level, the depressant effect on plasma cholesterol is sometimes lost and the plasma cholesterol returns towards the level before treatment. When attempts are made to maintain the lower plasma cholesterol level by increasing the dose of the thyroactive compound, the BMR may rise. Thus, the duration of the administration of the compound must also be taken into account in any assessment of the plasma-lipid and metabolic responses. This dissociation of cholesterol depression from elevation of the BMR can be accomplished with all these thyroactive compounds. However, it may be more apparent than real and

TABLE III. Approximate Minimal Daily Dose of Thyroid Compound Required to Reduce Plasma Cholesterol in Euthyroid Patients

Thyroactive compound	Approximate minimal daily dose	Additional references
3:5:3′:5′-Tetraiodo-L-thyronine (L-thyroxine)	200–600 μg.	Boyd & Oliver, 1957; Beierwaltes & Ruff, 1958; Doniach, Hudson, Trotter & Waddams, 1958
3:5:3′:5′-Tetraiodo-D-thyronine (D-thyroxine)	15–20 mg.†	
3:5:3′:5′-Tetraiodothyroacetic acid (TETRAC)	8–12 mg.	Goolden, 1956
3:5:3′:5′-Tetraiodothyroformic acid	200–300 mg.†	
3:5:3′:5′-Tetraiodothyropropionic acid*	3–6 mg.	Bauer, McGavack & Swell, 1959
3:5:3′:5′-Tetraiodothyro-amine	30–40 mg.	
3:5:3′-Tri-iodo-L-thyronine	60–80 μg.	Oliver & Boyd, 1956; Boyd & Oliver, 1957; Delmez & Engel, 1957; Doniach *et al.* 1958; Strisower, Gofman, Strisower & Delalla, 1958
3:5:3′-Tri-iodo-D-thyronine	300 μg.†	
3:5:3′-Tri-iodothyroacetic acid (TRIAC)	3–5 mg.	Oliver & Boyd, 1956; Trotter, 1956; Boyd & Oliver, 1957; Oliver & Boyd, 1957; Mackay, Goble & Sparkes, 1957; Menzies & Cooper, 1957; Doniach *et al.* 1958
3:5-Di-iodo-L-thyronine	10–15 mg.†	
3:5-Di-iodo-D-thyronine	150–200 mg.†	
3:5-Di-iodothyroacetic acid	100–150 mg.†	

* The propionic acid analogue of thyroxine (see footnote to Table I).

† The evidence for selection of the dose shown in this table will be published shortly by us.

depends partly on the inadequacy of the BMR to reflect minor changes in oxygen requirements of relatively small organs, such as the heart. This is one explanation of the observation that these compounds often increase the tendency to angina without raising the BMR (Oliver & Boyd, 1959).

5. Discussion

The animal studies outlined in this review are far from comprehensive, but even these limited assays demonstrate that a few carefully chosen tests can indicate the analogues of thyroxine worthy of further clinical appraisal of their effect on the plasma lipids. These studies in animals and also the subsequent clinical studies have shown that compounds iodinated at the 3:5:3′-positions are the most active in all respects. Similarly, in molecules exhibiting optical activity, the *laevo*-isomer is more active than the *dextro*-isomer. The relative potency of the thyroxine analogue in any one assay is not always a true measure of its clinical usefulness. For example, in ischaemic heart disease equal activity in all the tests may not be desirable; in this case, the usefulness of an analogue depends on its producing a greater effect on serum cholesterol than on the myocardium. In this respect, the *dextro*-isomers are more favourable than the *laevo*-isomers. All thyroxine analogues studied so far have affected the heart as well as the plasma cholesterol concentration. Although there is often a fairly wide margin between the dose required to lower cholesterol and the dose which results in myocardial effects, the tendency to angina was increased in a proportion of subjects by all analogues studied.

Interest in these thyroactive compounds arises mainly from the association of hypercholesterolaemia with coronary heart disease. This association has led to attempts to find an ideal depressant of plasma cholesterol. Although there is insufficient evidence to show that reduction of plasma cholesterol is beneficial to patients, it is necessary to establish whether prolonged cholesterol depression can influence morbidity and mortality. Experiments are therefore required to study diets and substances which depress the plasma lipids without side-effects. Current clinical studies of the effects of thyroxine analogues on the plasma lipids indicate that certain analogues may be of therapeutic value in this respect, and their clinical potentialities must be explored further.

ACKNOWLEDGEMENTS

We wish to express our thanks to the Scottish Hospitals Endowment Research Trust for a generous research grant and to Dr A. Rae Gilchrist for his continued interest and co-operation in this work.

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TRANSPORT OF THYROID HORMONES

J. R. TATA* D.Sc.

National Institute for Medical Research
Mill Hill, London

1 Methods for studying binding of thyroid hormones in blood
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  b Electrophoretic analysis
  c "Stabilization" of thyroxine
2 Thyroxine-binding protein (TBP)
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  b Distribution
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3 Physiological significance of the transport of thyroid hormones
  a Free and bound thyroxine
  b Fluctuations in the level of circulating TBP
  c Biological potencies of thyroxine and tri-iodothyronine
4 Conclusion
References

Our present-day knowledge of the transport of thyroid hormones can be said to begin with Trevorrow's observation that thyroxine added to human blood plasma was non-dialysable and could be precipitated with plasma proteins (Trevorrow, 1939). However, most of the information has been obtained only in the last eight years, after the identification of L-thyroxine and 3:5:3′-tri-iodo-L-thyronine as the two circulating thyroid hormones (Taurog & Chaikoff, 1947; Gross & Pitt-Rivers, 1952; see Pitt-Rivers, 1957; Pitt-Rivers & Tata, 1959). The aim of this review is to fit together and give a physiological meaning to the various, often fragmentary, facts related to the particular manner in which thyroid hormones are carried in the blood from the thyroid gland to tissue cells.

* Beit Memorial Fellow

1. Methods for Studying Binding of Thyroid Hormones in Blood

These methods have evolved with the improvements in analytical techniques of separation and identification of different blood constituents, especially of serum proteins. Much of the interpretation of facts depends on the particular method used. It is for this reason that the merits and disadvantages of the principal methods available for studying binding of thyroid hormones in blood will be considered first.

a. Chemical Fractionation of Plasma Proteins

Early attempts to localize the proteins with which thyroid hormones are precipitated involved the determination of iodine content of plasma proteins separated by chemical methods. At first serum albumin was thought to be the richest iodine-containing fraction (Salter, 1944; Taurog & Chaikoff, 1947), but with the use of ethanol for plasma protein fractionation, introduced by Cohn and colleagues (Cohn *et al.* 1950), Schmid (1953a) demonstrated that the Cohn Fraction VI-3 which is rich in α-globulins is the carrier of thyroid hormone. Such a method has the disadvantages of the difficulty of accurately determining small amounts of iodine present and the possibility that organic solvents used in protein fractionation might remove thyroxine during the procedure.

On the other hand, the study of thyroxine-binding by chemically separated plasma proteins is a useful step towards isolating the binding substance (or substances). For this purpose its combination with the methods of electrophoretic separation and thyroxine stabilization, described below, is more suitable. Thus, using electrophoretic analysis, Freinkel, Dowling & Ingbar (1955) were able to demonstrate that most of the thyroxine-binding activity of human plasma was located in Cohn Fractions IV-4, IV-6 and IV-9. Any of these fractions would therefore be suitable as starting material for further purification.

b. Electrophoretic Analysis

Gordon, Gross, O'Connor & Pitt-Rivers (1952) were the first to report the distribution of ^{131}I-labelled thyroid hormone in human serum on paper electrophoresis. The combination of localization by radioactivity and separation by paper electrophoresis is now the most widely used method, and several authors have shown that most of the circulating thyroid hormone migrates to a position between α_1- and α_2-globulins (inter-alpha zone) (Robbins & Rall, 1952; Larson, Deiss & Albright, 1952; Horst & Rösler, 1953). A

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similar mobility was observed in free electrophoresis (Robbins & Rall, 1957). This binding component has been commonly designated as "thyroxine-binding protein" (TBP) or "thyroxine-binding globulin" (TBG), although it may be composed of more than one protein.

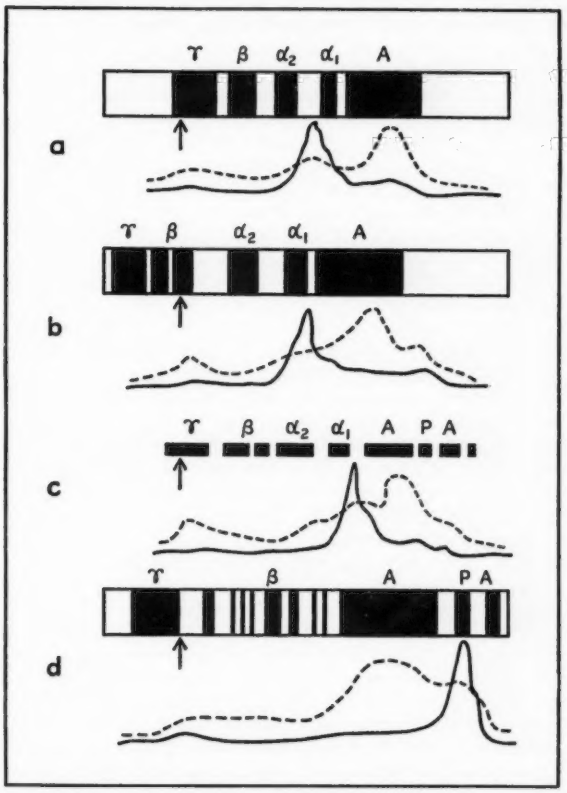
It should be emphasized that no protein has yet been isolated which can account for the binding of thyroid hormone in blood; however, the term "thyroxine-binding protein" or "TBP" will be used in this discussion for the sake of conformity and to avoid another definition for something not yet isolated. It will generally be used to denote the protein fraction with a mobility between that of the α_1- and α_2-globulins in free and paper electrophoresis in buffers of ionic strength not exceeding 0.1. Further, this protein fraction also binds tri-iodothyronine and other related substances, although the interaction with thyroxine has been studied in greater detail. Very often a small amount of thyroxine migrates with the albumin zone; in most cases the albumin-bound fraction increases as the level of thyroid hormone is raised.

For most studies on TBP, zone electrophoresis has been performed on horizontally arranged filter-paper in the presence of veronal buffer at pH 8.6, $\mu = 0.05$–0.1. Robbins (1956) has introduced the "reverse-flow" method which permits a better quantitation of distribution of radioactive thyroxine present in the inter-alpha and albumin zones. However, with the introduction of newer supporting media such as modified celluloses, agar and starch gels and the variation in buffers, TBP has been localized in zones other than that intermediate between α_1- and α_2-globulins. The influence of electrophoretic supporting media on the mobility of TBP is illustrated in fig. 1.

Another factor which seems to determine the migration and homogeneity of the thyroxine-rich zones is the nature and ionic strength of the electrophoresis buffer, besides the obvious changes expected from differences in pH. Thus, recently Ingbar (1958) reported that in tris (2-amino-2-hydroxymethyl-propane-1:3-diol) buffer of high ionic strength ($\mu = 0.2$), thyroxine added to normal unmodified plasma migrated not only in the inter-alpha zone on paper, but a part of it also moved to a zone ahead of the albumin (pre-albumin zone). It has not yet been shown that the substance moving to the pre-albumin zone under such conditions is identical with substances moving ahead of the albumin on other media (starch gel, acid cellulose) with buffers of low ionic strengths, or with isolated pre-albumin preparations. Until this is done, caution should be exercised in comparing the above diversity with thyroxine-binding properties of an isolated pre-albumin (Schultze, Schönenberger & Schwick, 1956), as demonstrated by Tata (1959b, 1959c).

If the above variables are taken into account, electrophoretic analysis has many advantages, and paper electrophoresis has been the most widely used technique in the study of thyroxine-binding in health and disease. The major advantage is the possibility of localizing the binding component (or components) (depending on the resolution); hence it is most useful in determining the relative distribution of thyroxine on primary and secondary binding components in serum, i.e., TBP and albumin. Within certain limits, electrophoretic analysis is also useful for determining the relative binding capacities of TBP and albumin in different samples of blood. This has been done either by determining the saturation point of TBP by adding increasing amounts of thyroxine or related substances, or by making TBP compete for the

FIG. 1. Influence of Supporting Medium on the Electrophoretic Mobility of Thyroxine-Binding Protein in Human Serum



a: paper or cellulose acetate (veronal buffer, pH 8.6)
b: agar gel (phosphate-borate buffer, pH 8.2)
c: cellulose treated with acid and ethanol (material packed as a column, veronal buffer, pH 8.6)
d: starch gel (borate buffer, pH 8.4)
The dark bands represent the zones of distribution of serum proteins.
α_1, α_2, β, γ: globulins
A: albumin
PA: pre-albumin
The curves under these bands denote the distribution of radioactivity when ^{131}I-labelled thyroxine was added to serum in the final concentration of 1.5×10^{-7} M (solid line) or 1.5×10^{-6} M (broken line). Vertical arrows show point of application of sample.

hormone against increasing amounts of added albumin (Robbins & Rall, 1955, 1957; Freinkel *et al.* 1955; Myant, 1957; Myant & Osorio, 1959). However, the high adsorption power of paper for thyroxine can become a major handicap when accurate quantitative determinations are to be made or if only very small amounts of protein are available for examination.

c. "Stabilization" of Thyroxine

This method, developed recently, is based on the transient instability of thyroxine and related iodophenols, revealed by spectrophotometric and chromatographic evidence, under conditions which lead to the ionization of their phenolic

groups (Tata, 1959a). Certain proteins inhibit this instability, and the effect has been termed stabilization of thyroxine and related substances (Tata, 1959b). The extent of such a stabilization is directly related to the concentration of the proteins and their thyroxine-binding affinity. Thus, by measuring stabilization of thyroxine at increasing concentrations of proteins (at the same concentrations of thyroxine), it is possible to derive relative thyroxine-binding powers of various biological fluids and isolated proteins. The slope of a curve thus obtained gives the relative affinity. This method is more accurate than paper electrophoresis, especially at low protein concentrations (as in cerebrospinal fluid and amniotic liquor), when the interference due to adsorption of thyroxine on paper would be particularly marked. If similar measurements are made with increasing concentrations of thyroxine added, the shift in the intercepts on the abscissae indicates the binding capacity as distinct from affinity.

The stabilization method also makes possible the measurement of thyroxine-binding at a physiological pH; this is important in view of the dependence of binding power on the H^+-ion concentration of the medium (Tata, 1959b). The disadvantage of this method is that it is not possible to distinguish between the thyroxine-binding powers of the individual components in a mixture. In this respect the combination of the technique with electrophoretic separation should prove more fruitful.

2. Thyroxine-Binding Protein (TBP)

a. Nature

Since TBP or any other plasma component with high thyroxine-binding power has not yet been isolated, nothing definite can be said as to its nature. Earlier studies suggested that it might be a glycoprotein, and, consistently with the low isoelectric point of some acidic α_1-glycoproteins (Mehl, Golden & Winzler, 1949; Schmid, 1953b; Schmid, Bencze, Nussbaumer & Wehrmüller, 1959), TBP is negatively charged at pH 4.5 with an electrophoretic mobility of -1.7×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ (Robbins, Petermann & Rall, 1955). The mobility at pH 8.6 is -4.7×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. These values were derived by determining the mobility of the major radioactive fraction in human serum to which a trace amount of ^{131}I-labelled thyroxine was added. Similarly, when the sedimentation constant was determined in a partition cell of the ultracentrifuge, a value of $S_{20,w} = 3.3$ was obtained for the major thyroxine-carrying component. If the error involved in such measurements is considered, it is conceivable that TBP might be one of the α_1-glycoproteins described by Schmid (1953b) or by Schultze and his colleagues (Schultze, Göllner, Heide, Schönenberger & Schwick, 1955).

As we have already seen in fig. 1, TBP is found to migrate in the pre-albumin zone on starch-gel electrophoresis (Rich & Bearn, 1958; Allison, 1959). But the mobility of acidic α_1-glycoprotein as one of the pre-albumin zones in human serum when analysed with starch-gel electrophoresis (Poulik & Smithies, 1958) does not invalidate the assumptions derived from free or paper electrophoresis. On the other hand, the data of Ingbar (1958) with paper electrophoresis in tris buffer of high ionic strength suggest that TBP, or the protein with a mobility between that of the α_1- and α_2-globulins, and a pre-albumin are two distinct and principal thyroxine-binding proteins of human serum. The introduction of a second strong TBP of unknown binding power would mean a revision of some of the data on binding affinity and capacity of TBP. However, a possible solution could be found in the demonstration of Tata (1959c) that a large fraction of Schultze's human serum pre-albumin is probably present as a complex in the α-globulin zone in paper electrophoresis in buffers of the usual low ionic strength. High ionic strength or the "molecular sieve" effect of starch gel as a medium could cause a part or whole of this complex to split. But it is still likely that the TBP and a pre-albumin complex could represent different substances migrating in the α-globulin zone in ordinary paper electrophoresis. Hence, only the isolation from serum of a substance with all the properties of TBP can help in deciding its nature.

b. Distribution

TBP, as the α-globulin-like protein fraction with high thyroxine-binding power, is distributed in the blood of a large variety of animals (Robbins & Rall, 1957; see Pitt-Rivers & Tata, 1959). These range from fish to reptiles, amphibia and all the mammals studied. Notable exceptions are chicken and duck bloods in which no TBP, or only traces of it, could be found (Tata & Shellabarger, 1959). In these birds, thyroxine is bound principally to serum albumin in relatively loose association. The lack of TBP in these species will be considered later in discussing its physiological significance. Another interesting observation has been reported by Myant & Osorio (1959) on the TBP in the serum of foetal and new-born animals. These authors have shown that rabbit foetal serum contains a TBP which is different from adult TBP; the foetal component migrates between β- and α_2-globulins, while adult rabbit serum TBP is located between α_1-globulin and albumin in paper electrophoresis. Foetal TBP is first observed in 19-day-old foetuses and is slowly replaced by the adult-type TBP in the new-born rabbit. Although the thyroxine-binding capacity of foetal TBP is only one-tenth that of adult TBP, it has a fivefold higher affinity. This finding is of help in understanding the problem of passage of thyroid hormones from mother to foetus.

The adult-type serum TBP has also been detected in a variety of extravascular fluids (Alpers & Rall, 1955; Robbins & Rall, 1957; Freinkel, Ingbar & Dowling, 1957; Neuhaus & Sogoian, 1959). It is found in relatively large amounts in synovial, cerebrospinal and amniotic fluids (Tata, unpublished). These fluids also contain pre-albumin in proportionately larger amounts compared to those in the serum, but the significance of its association in thyroid hormone transport is not understood.

c. Properties

Although serum TBP has not been isolated, it is possible to study some of its binding properties in unfractionated serum or in protein fractions enriched by TBP. For some studies it is even preferable to study it in whole serum rather than in any isolated fraction. The most notable properties of TBP are its low capacity to bind thyroxine and its selective specificity in binding thyroxine analogues.

When increasing amounts of labelled thyroxine are added to serum, the radioactivity at first found in the inter-alpha zone is displaced to the albumin zone (Robbins & Rall, 1955; Albright, Larson & Deiss, 1955; Freinkel *et al.* 1955; Myant, 1957). Up to a concentration of about 0.10 μg./ml. of serum almost all of the thyroxine can be located in the TBP zone; between 0.50 μg./ml. and 0.70 μg./ml. it will be distributed

in about equal amounts between TBP and albumin, while between 1.0 μg./ml. and 7.0 μg./ml., albumin will account for over 90% of the thyroxine added (see fig. 1). Above this amount, thyroxine will be found associated with almost all the other serum proteins in a non-specific manner. These figures illustrate the very low capacity of TBP to hold on to thyroxine, whereas albumin has a high binding capacity. The ease with which TBP is saturated indicates that the amount in which it is present in serum must be very low, even if only one molecule of thyroxine were bound to each TBP molecule. On the other hand, the affinity of TBP for thyroxine is much higher than that for albumin. This is best seen by the stabilization method at low concentrations of thyroxine. A similar conclusion has been reached by Myant (1957) from studies in vivo which showed that thyroxine bound to albumin diffuses more readily into tissues. Robbins & Rall (1957) have calculated that the association constant of TBP and thyroxine is of the order of 7.9×10^9.

TABLE I. Relative Binding Affinity of Human Serum TBP for L-Thyroxine and its Analogues

Compound	Relative affinity
L-Thyroxine	100
D-Thyroxine	80
3:5:3′-Tri-iodo-L-thyronine	30
3:5-Di-iodo-L-thyronine	0
3:5:3′:5′-Tetrabromo-L-thyronine	25
3:5:3′-Tribromo-L-thyronine	10
3:5:3′:5′-Tetraiodothyroacetic acid	16
3:5:3′-Tri-iodothyroacetic acid	10
3:5-Di-iodo-L-tyrosine	<5

TBP exhibits a marked selective specificity in interacting most intensely with thyroxine. Table I demonstrates that any modification of the chemical structure of thyroxine will result in a reduced binding affinity. However, the two optical isomers of thyroxine are bound with about the same intensity. These measurements were made by using ^{131}I- or ^{82}Br-labelled analogues of thyroxine. In earlier studies, the relative binding affinities were determined from the amount of non-radioactive analogue of thyroxine to be added in order to displace a trace amount of radioactive thyroxine bound to the TBP (Larson & Albright, 1955; Robbins & Rall, 1955, 1957). The lower affinity of TBP for tri-iodothyronine explains the ease with which TBP can be saturated with respect to tri-iodothyronine. Serum albumin, on the other hand, binds both hormones about equally firmly (Tata & Shellabarger, 1959); the significance of this is considered below. Other binding properties have been described by Robbins & Rall (1957) and Pitt-Rivers & Tata (1959).

3. Physiological Significance of the Transport of Thyroid Hormones

In this section, an attempt will be made to derive some meaning in physiological terms for the manner in which thyroid hormones are carried in the blood. This aspect has been recently discussed in detail by Tata (see Tata, 1960).

a. Free and Bound Thyroxine

The displacement of thyroxine bound to TBP and its reversibility indicate that there exists a simple equilibrium between protein-bound and free thyroxine in the plasma. It is impossible as yet to measure the amount of unbound thyroxine, but a value for it has been derived by Robbins & Rall (1957). Since most of the circulating thyroxine can be measured as protein-bound iodine (PBI), a value of 5 μg./100 ml. (with an association constant of 7.9×10^9) gives the figure of 3.9×10^{-11} M as the concentration of free thyroxine in normal human serum. If the amount of TBP in blood were constant, the level of free thyroxine would be a function of the amount of PBI. In many cases of alterations of thyroid function which result in increased or decreased PBI, the amount of TBP is found to be unchanged (Albright *et al.* 1955; Robbins & Rall, 1957). If it is assumed that only free or non-protein-bound thyroxine (or tri-iodothyronine) can diffuse into tissue cells, then a larger amount of thyroid hormone will leave the blood in individuals with a high serum PBI, as in hyperthyroidism. Thus Robbins & Rall (1957) were able to observe a linear relationship between calculated values of free thyroxine and the daily degradation of hormonal iodine (which is a good indication of the amount of hormone entering into tissues) in normal, hypothyroid and hyperthyroid subjects.

There is now much evidence to indicate that only free thyroid hormones can leave the blood for entry into tissues. Most of it is deduced from studies in which the uptake of labelled thyroxine or tri-iodothyronine by erythrocytes and tissue slices is inhibited by serum, through the occurrence of binding by TBP (Freinkel *et al.* 1957; Crispell, Coleman & Hyer, 1957; Hogness, Lee, Berg & Williams, 1957; Hamolsky, Stein & Freedberg, 1957; Béraud, Cruchaud & Vannotti, 1958). There is no direct evidence to show that thyroxine or tri-iodothyronine can leave the blood in the protein-bound state.

b. Fluctuations in the Level of Circulating TBP

An increase in the level of circulating TBP would mean that, for the same amount of thyroxine or tri-iodothyronine in blood, the level of free hormone, and hence the amount available for tissues, would fall. If the output of thyroid hormone from the gland were not raised, then symptoms of hypothyroidism would soon become evident. The best example available for demonstrating the effects of a change in TBP level is a comparison of thyroxine-binding in the blood of hyperthyroid patients and pregnant women, which has attracted much attention recently (Heinemann, Johnson & Man, 1948; Dowling, Freinkel & Ingbar, 1956b; Robbins & Rall, 1957; Robbins & Nelson, 1958). From the data brought together in Table II, it will be seen that the PBI levels are raised both in hyperthyroidism and in pregnancy. However, it is only in hyperthyroidism that the peripheral effects of an increased thyroid hormone level in blood, such as increased basal metabolic rate (BMR), are noticed. This is because an unchanged level of TBP in the serum of a hyperthyroid individual allows more thyroid hormone to enter the tissues. In pregnancy there is a marked elevation of serum TBP, which may be due to the general elevation of α-globulins reported for this condition (Mack, 1955). The immediate (but unnoticed) effect of this elevation would be a drop in the free or diffusible thyroxine, which would then lead to a stimulation of the thyroid gland via the pituitary (described by Brown-Grant, p. 165 of this number of the Bulletin) and consequently a high PBI. Hence the elevated PBI in pregnancy, unlike that in hyperthyroidism, is the result of a compensatory

TABLE II. Thyroid Function, Basal Metabolic Rate, Circulating Thyroxine Concentration and Thyroxine-Binding Capacity of Thyroxine-Binding Protein in Normal, Hyperthyroid and Pregnant Subjects

Condition	Thyroid function	Basal metabolic rate	Serum thyroxine* (μg./ml.)	Thyroxine-binding capacity (μg./ml.)
Normal	Normal	Normal	0.10 ± 0.03	0.19 ± 0.04
Hyperthyroidism	Elevated	Elevated	0.27 ± 0.08	0.18 ± 0.04
Pregnancy†	Elevated	Normal	0.23 ± 0.06	0.48 ± 0.05

* Derived from protein-bound iodine
† Maximum values at 12–36 weeks of pregnancy

mechanism to maintain a normal level of free thyroxine for the tissues.

The level of TBP and thyroid activity can be artificially elevated by oestrogen treatment in both men and women (Dowling, Freinkel & Ingbar, 1956a; Robbins & Nelson, 1958; Jensen, 1959). Recently Tanaka & Starr (1959b) have described several cases of non-pregnant women with high PBI values (9–30 μg./100 ml.) but normal BMR (−14 to +11). An elevation in the level of circulating TBP is described by these authors as the cause of this anomaly. Vannotti & Béraud (1959) conclude that the high PBI level in infectious hepatitis is due to an increased serum TBP level which could explain the lack of symptoms of hyperthyroidism and slower turnover of thyroxine in this disease. An example of the opposite effect is a case of a clinically euthyroid subject with a low PBI level; in this subject no TPB could be detected but most of the added thyroxine was bound to albumin (Tanaka & Starr, 1959a). As mentioned earlier, albumin binds thyroxine less firmly than does TBP, thus favouring a higher level of free thyroxine.

c. Biological Potencies of Thyroxine and Tri-Iodothyronine

Tri-iodothyronine is found to be about 3–6 times as active as thyroxine in a wide variety of potency tests in all the mammals studied and also in amphibian metamorphosis (see Pitt-Rivers & Tata, 1959). However, Shellabarger (1955) and Newcomer (1957) failed to observe this higher potency when the assays were carried out in the chicken. An explanation has now been offered by Tata & Shellabarger (1959) to account for this difference in response to the two thyroid hormones on the basis of differences in serum thyroxine-binding patterns. In essence, it states that the variations in the biological potencies of thyroxine and tri-iodothyronine are determined by whether the major thyroxine-binding component in serum does or does not bind thyroxine more firmly than tri-iodothyronine.

The major piece of evidence for the above conclusion was the virtual absence of a mammalian TBP-like component from the blood of chicken and duck. Most of the thyroid hormone in bird serum is bound to albumin. Mammalian and avian albumins bind thyroxine and tri-iodothyronine almost equally firmly, whereas TBP will bind thyroxine 3–4 times more firmly than tri-iodothyronine. When the disappearance rates of the two hormones were measured, they were identical in the chicken, whilst in mammals tri-iodothyronine has a biological half-life 2–4 times shorter than thyroxine. Results of these studies in man, rat and chicken are summarized in Table III. Thus it follows that the biological potencies of thyroxine and tri-iodothyronine reflect the relative ease with which they can diffuse into the tissues.

TABLE III. Physiological Potencies, Biological Half-Lives ($T_{\frac{1}{2}}$) and Protein-Binding of Thyroxine (T_4) and Tri-Iodothyronine (T_3) in Man, Rat and Chicken

(Figures expressed as ratio of T_3:T_4)

Species	Physiological potency	Biological $T_{\frac{1}{2}}$	Major T_4-binding and T_3-binding component in serum	Relative binding affinity
Man	3–5	0.3–0.4	Thyroxine-binding protein	0.25–0.35
Rat	2–6	0.3–0.5	Thyroxine-binding protein	0.25–0.28
Chicken	0.8–1.0	1	Albumin	0.8–0.95

The above explanation is the first proof that biological potencies (or, more correctly, the speeds of action) of two structurally related hormones are dependent on the manner in which they are transported from their site of production to their sites of utilization. This idea is in opposition to the widely held view that these differences can be explained only on the basis of their action on enzymes. In fact, in the case of thyroid hormones, all attempts to demonstrate a more pronounced effect of tri-iodothyronine at the cellular or enzymic level have ended in failure (see Pitt-Rivers & Tata, 1959). It may well be that diffusibility into tissues of other structurally related substances with biological activity determines their relative potencies, especially when their action on enzymes fails to give the answer.

4. Conclusion

Thyroid hormones in blood are firmly bound to thyroxine-binding protein (TBP). Although TBP has not been isolated, it is possible to study its nature and some of its properties by a variety of methods now available. The increase in thyroid activity without concomitant increase of BMR during pregnancy has been explained on the basis of an increase in circulating TBP. An important physiological aspect of the study of thyroid hormone transport is the hypothesis that the biological potencies of thyroxine and tri-iodothyronine are governed by the affinity with which the two hormones are bound to TBP.

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GENETIC FACTORS IN THYROID DISEASES

F. D. KITCHIN M.D. M.R.C.P.

W. HOWEL EVANS M.D. M.R.C.P.

Thyroid Clinic
David Lewis Northern Hospital, Liverpool

Department of Medicine
University of Liverpool

1 Toxic diffuse goitre
2 Genetic aspects of the hypothyroid states
3 Simple goitre
4 Role of the phenylthiocarbamide taste response in thyroid disease
References

The familial incidence of thyroid disease has been recognized for many years, but classical genetic analyses (studies of pedigrees and of twins) based on Mendelian principles have elucidated only a few of the problems. Thus a more or less definite method of inheritance has been shown to occur in toxic diffuse goitre (Graves's disease, primary thyrotoxicosis) and also in the rarer conditions of familial goitrous cretinism, congenital deafness with goitre and certain instances of athyreotic cretinism. However, no genetic factors have been revealed by the above-named methods of investigation in carcinoma of the thyroid and the various forms of thyroiditis; but there is indirect evidence that adult myxoedema is in part conditioned by hereditary influences. The outstanding difficulty, which remains as yet unsolved, concerns the problem of simple goitre—in which the parts played by environmental and genetic factors are still unresolved.

Recently attention has been directed to the investigation of human polymorphic characters in relation to disease, for example the ABO blood-group frequencies[1] and the secretor status[2] in peptic ulceration. One such example of human polymorphism is taste "blindness" to phenylthiocarbamide (PTC, or phenylthiourea). Since PTC and related compounds (thiocarbonamides) exhibit a marked antithyroid activity, as well as the specific differential taste response, this gene marker has been used in an investigation of hereditary factors in thyroid disease. The two separate surveys referred to in section 4 have now shown a significant association between the incidence of the taster gene for PTC and thyroid disease and there is little doubt that the PTC genes are intimately concerned with the determination of the type of goitre an individual may develop.

This recent advance and the background of previous knowledge concerning hereditary factors in thyroid disease will now be considered in more detail.

[1] See Fraser Roberts, *Brit. med. Bull.* 1959, **15**, 129.—ED.

[2] People can be divided into two types according to whether they do or do not secrete antigens of the ABO group in their saliva; a person's secretor status is genetically determined, secretion being dominant to non-secretion.—ED.

1. Toxic Diffuse Goitre

Long-continued investigation has failed to reveal any extrinsic factor of aetiological importance to account for the familial disposition in toxic diffuse goitre, although much has been learnt of the functional disturbance in this disorder. In a situation of this sort, pedigree analysis can be expected to show whether genetic influences exert a major part, but in a common disease single families or small groups of families provide an unsuitable basis for an investigation of the manner of inheritance. Thus Bartels (1941) found slender evidence from many published pedigrees for a dominant mode of inheritance, with incomplete penetrance and sex-limitation to women, but because of the uncertainty of the diagnosis and the lack of a control group he considered this material unfit for detailed analysis. In his own observations, derived from 204 propositi[3] with Graves's disease and 21 propositi with toxic adenoma, the control group consisted of 498 normal propositi who were regarded as representative of the Danish community. A total familial incidence of 47% was found in patients with thyroid disease, but 60% in those with Graves's disease alone. This fact was explained by the supposition that Graves's disease was always hereditary, whereas toxic adenoma could be either hereditary or exogenous in origin. In addition, Bartels found that relatives of patients with Graves's disease showed a greater frequency of simple goitre and myxoedema, as well as a greater incidence of Graves's disease itself.

Thus the gene or gene complex which leads to the development of toxic diffuse goitre also manifests itself just as often, or more so, in other forms of thyroid disease. Bartels concluded, after extensive genetic argument, that the inheritance in Graves's disease was controlled by a single recessive gene of variable expressivity and with relative sex-limitation to women, in whom there was a penetrance of 70–80%.

Martin & Fisher (1945) likewise studied the hereditary and familial aspects of exophthalmic goitre (toxic diffuse goitre) in a series of 90 cases in adults. The genetic evidence pointed strongly to the inheritance of a single recessive characteristic favourable to the disease and perhaps necessary for its occurrence, since the proportion of affected siblings in these families greatly exceeded that of the parents. On this interpretation, not all those who are homozygous for the recessive gene developed the disease, and in this series only half the females and a quarter of the males with the recessive gene manifested symptoms; this finding may be taken to indicate the existence of modifying factors.

Thus in toxic diffuse goitre the existence of a genetic abnormality is demonstrated that favours the development of the disease in the presence of some other factor, either internal or external. The way in which this genetic abnormality produces the wide-spread functional disturbances in Graves's disease is clearly not revealed in investigations of this kind. Nevertheless, the conclusion is not a restatement of Warthin's constitutional theory of nervous instability (Warthin, 1928), since it is based on Mendelian principles and not on ill-defined clinical impressions.

Trotter (1959) makes the interesting suggestion that further genetic investigation of Graves's disease should be concerned with abnormalities such as exophthalmos, vitiligo and local-

[3] Propositus: a modern adaptation of the participle of *proponere*, used for the first of a family to show some condition which on investigation proves to be familial.—ED.

ized pretibial myxoedema, which are not in themselves the direct consequence of hyperthyroidism. He also suggests (on the basis of the work of Ingbar, Freinkel, Dowling & Kumagai (1956)) that augmented thyroxine metabolism in the absence of clinical hyperthyroidism, demonstrable in relatives of patients with Graves's disease, might be the primary disturbance of function.

2. Genetic Aspects of the Hypothyroid States

From a genetic standpoint there are three main varieties of hypothyroidism to be considered: (i) endemic cretinism; (ii) sporadic cretinism, comprising familial goitrous cretinism and athyreotic cretinism; and (iii) adult myxoedema.

Endemic cretinism is not associated invariably with endemic goitre.[4] In Switzerland, in contrast to South America, the occurrence of these two conditions in the same localities led to the belief that both were caused by an environmental deficiency of iodine. This view was rationalized by assuming that the stress of years of simple goitre in repeated generations of the forbears had so weakened the functional capacity of the thyroid that a state of cretinism resulted finally in the progeny. Clements (1958) reviewed critically the evidence for this established belief and came to the conclusion that the association depended on factors other than iodine deficiency. He emphasized that it was known that endemic cretinism was often restricted to villages and even households within mountainous regions and that it bore a direct relationship to the degree of consanguinity in these areas. Geographical and social conditions of this sort are known to favour the accumulation of deleterious recessive genes. In Clements' view the opening up of communications and the consequent redistribution of population were probably just as important factors in the reduction of the amount of cretinism as the introduction of iodine prophylaxis, since wider social contacts reduced the extent of intermarriage. Finally, he felt that, as an explanation of the findings in endemic goitre, the theory of the inheritance of acquired characteristics bore too much resemblance to Lamarckism to be acceptable today, and that a thorough genetic survey should be made.

Familial goitrous cretinism has been the subject of much recent physiological research, and three main types of biochemical defect have been differentiated. In many of the examples studied, parental consanguinity has been found and, in a particular family reported by Hutchison & McGirr (1956), the genetic evidence points strongly to a recessive mode of inheritance. The biochemical aspects of this subject are considered more fully by McGirr on p. 113 of this number of the Bulletin and, in general, from all the available genetic data, it appears that familial goitrous cretinism is inherited as an autosomal recessive character.

Athyreotic cretinism is the usual type of cretinism seen in areas where simple goitre is not endemic and in most cases there is no family predilection (Stanbury & Querido, 1956). However, Ainger & Kelley (1955) described three siblings with cretinism in one family—two without thyroid enlargement and one with slight enlargement. The parents were cousins, and a genealogical survey revealed at least 12 additional related persons who may have been similarly affected. The family data in this particular instance are consistent with the hypothesis that the defect was inherited in a recessive manner.

In the case of adult spontaneous myxoedema there is little evidence available concerning hereditary factors.

Bartels (1941), after a study of the literature, was unable to say anything definite on the matter. Nevertheless he felt that, although the manner of inheritance could not be deduced with any certainty, there was sufficient evidence to show that myxoedema had the same familial disposition as other thyroid affections and was not infrequently observed in the relatives of patients with Graves's disease. The aetiology of spontaneous myxoedema still remains an enigma, but recent evidence of the existence of an auto-immune response in this disease and also in lymphadenoid goitre may indicate that the two conditions are related. It is clear that a comprehensive study of genetic factors in spontaneous myxoedema is needed and that this study should include examples of lymphadenoid goitre.

3. Simple Goitre

The term "simple goitre" is used to include those series of pathological changes, often in response to iodine deficiency, which result from interference with normal hormone production. The compensatory mechanism which is evoked produces at first a diffuse and later a focal hyperplasia in the gland. In spite of these structural changes, the majority of persons with simple goitre are euthyroid, though in a certain number symptoms of hyperthyroidism develop in later life (toxic adenomatous or nodular goitre—"secondary thyrotoxicosis"). In the minority hypothyroidism readily occurs, as may be seen in familial goitrous cretinism or the simple goitre which results from intake of antithyroid substances.

Endemic goitre, it is generally agreed, is due mainly to external factors. Although the iodine deficiency theory has been confirmed by a host of investigators, not all simple non-toxic goitrous states can be explained in this fashion and even in endemic regions not all persons are affected. In addition there is a marked female predisposition to the disease. Thus in hyperendemic regions the ratio of females to males approaches 1:1, whereas in regions of sporadic goitre this may exceed 10:1. It follows therefore that internal factors must play a part in the genesis of the disease and that their influences will be recognized more easily in non-endemic regions.

According to Bartels (1941) hereditary factors may assert themselves, either in a direct manner by developing a certain quality (as for example in polydactyly) or in an indirect manner by impairing the response of an organ to variations in external or internal conditions. He reasoned that the latter method must be the explanation for the smooth transition from regions of endemic to those of sporadic goitre, and for the sex differences in areas of sporadic disease. Thus, in some ways the functional adaptability of the thyroid is restricted in its endeavour to meet the demands of changing circumstances.

Genes are known to control biochemical processes and, since for many characters the heterozygotes are biologically fitter than either homozygote, there is a strong tendency for man to develop the biochemical complexity that is the basis of functional adaptability. By this reasoning it is evident that, in areas throughout the world where the majority of the population have goitre, conventional genetic studies are valueless as these differences are overwhelmed by environmental iodine deficiency.

Bartels (1941), from a review of the literature concerning hereditary factors in thyroid disease, found that little study

[4] See Trotter, p. 92 of this number of the Bulletin.—Ed.

had been made of sporadic goitre. His conclusion indicated that sporadic goitre could be hereditary and transmitted as a dominant gene with incomplete penetrance and relative sex-limitation to women. He treated this finding with the greatest reserve since many of the studies had been made in mildly endemic regions, and generally concerned either single families or groups of families, without adequate controls.

A large family study was made by Martin & Fisher (1945) who investigated 111 examples of adenomatous goitre both toxic and non-toxic. They found no evidence of an inherited trait in nodular goitre, but added that the possibility could not be ignored. In 1951 the same authors re-investigated the hereditary factors in toxic adenomatous goitre (Martin & Fisher, 1951), since Bartels' data in this category consisted of only 21 propositi. Bartels (1941) had found from his small series of pedigrees that toxic adenoma could be either hereditary or non-hereditary, according to the nature of the pre-existing goitre. However, Martin and Fisher were unable to confirm this dual view and considered that this was supporting evidence for the clinical distinction between the two main varieties of thyrotoxicosis.

It seems clear from the results of these well-conducted investigations that, if a hereditary factor in simple goitre exists, it is complex and unlikely to depend solely upon the effect of a single gene.

4. Role of the Phenylthiocarbamide Taste Response in Thyroid Disease

The previous investigations have used pedigree analysis to demonstrate Mendelian principles in thyroid disease. However, it is possible to tackle the problem from another direction. Since 1931 (Fox, 1931) it has been known that some people—approximately 30% of European populations—cannot taste PTC, while the remainder can. In 1932 Blakeslee discovered that the inability to taste PTC was inherited as a Mendelian recessive character (genotype *tt*) and that the ability to taste was dominant (genotype *TT*, *Tt*). Further investigation (Das, 1958; Brandtzaeg Merton, 1958) has largely confirmed this hypothesis, though there is very occasionally incomplete penetrance of the taster gene.

In 1949, Harris & Kalmus pointed out that the specific chemical grouping which is concerned in this human dimorphism is common to a class of antithyroid substances (thiocarbonamides) both natural and synthetic. In view of this association these authors, together with Trotter (Harris, Kalmus & Trotter, 1949), examined the taste reaction to PTC of patients suffering from various forms of thyroid disease (see Table I). They found a significantly high incidence of non-tasters among patients with nodular goitre and thought that non-tasters of PTC might be more susceptible to the development of nodular goitre.

TABLE I. Phenylthiocarbamide Taste Reactions in Toxic Diffuse Goitre, Adenomatous Goitre and Controls (Harris, Kalmus & Trotter, 1949)

Condition	Total	Non-tasters	
		Number	Percentage
Normal	541	169	31.2
Toxic diffuse goitre	218	67	30.7
Nodular goitre (61% non-toxic)	134	55	41.0

TABLE II. Phenylthiocarbamide Taste Thresholds in Diffuse Goitre, Adenomatous Goitre and Controls (Kitchin, Howel-Evans, Clarke, McConnell & Sheppard, 1959)

Condition	Total	Non-tasters	
		Number	Percentage
Normal	265	78	29.4
Adenomatous goitre (toxic and non-toxic)	246	97	39.4
Toxic diffuse goitre	133	22	16.5

Kitchin, Howel-Evans, Clarke, McConnell & Sheppard (1959) conducted a more detailed survey and confirmed that in 246 patients with adenomatous goitre, both toxic and non-toxic, there was a significantly high incidence of non-tasters (genotype *tt*), and that this was greater in men than in women. A surprising feature of this investigation was the finding of a highly significant deficiency of non-tasters in the group of patients with toxic diffuse goitre (see Tables II and III). In addition, the ABO blood groups and secretor status were examined in this series of patients and found to be normal.

If a causal hypothesis is accepted as an explanation of these findings, then the results indicate that the taster and non-taster genotypes are important in determining the type of thyroid disease an individual may develop, and that in nodular goitre the genotype is about twice as important in males as in females. That is to say, the genes determining the taste response to PTC act by modifying the expression of the gene or genes for thyroid disease.

The taste response to PTC probably reflects a wide fundamental biochemical divergence (Harris, 1955), and it is known that thiocarbonamides block the intra-thyroidal synthesis of thyroxine (Astwood, 1943). If non-tasters of PTC are assumed to be more susceptible to this action, then, provided that the stimulus is sufficiently sustained, a nodular goitre will develop in due course. A naturally occurring thiocarbonamide, L-5-vinyl-2-thio-oxazolidone (goitrin), has been found in members of the genus *Brassica*, but the conditions of chemical extraction indicate that cooking destroys the enzyme system responsible for its liberation from a precursor in the macerated vegetable (Greer, Ettlinger & Astwood, 1949). However, vegetables of this class are fed to animals as winter

TABLE III. Distribution of Taste Responses in Males and Females with Adenomatous Goitre, Toxic and Non-Toxic

Condition	Total	Non-tasters	
		Number	Percentage
Adenomatous goitre (toxic and non-toxic)	Females 218	79	36.2
	Males 28	18	64.3

fodder and there is evidence that cow's milk in the winter months contains an antithyroid substance with similar thyroid-blocking properties to the thiocarbonamides (Greene, Farran & Glascock, 1958). In addition Clements & Wishart (1956) described an outbreak of simple goitre in Tasmania in spite of adequate iodine prophylaxis; they thought this was due to the goitrogenic effect of milk from cows fed on one of the *Brassica* (marrow-stemmed kale)[§].

On this basis it is difficult to construct a hypothesis to account for the low incidence of non-tasters in toxic diffuse goitre, unless it is assumed that homozygous tasters (genotype *TT*) are even less susceptible to the action of thiocarbonamides than non-tasters (*tt*) or heterozygous tasters (*Tt*). This decreased susceptibility might then remove one of the normal restraining mechanisms in thyroid homeostasis and result in a gland which might react more vigorously to extra-thyroidal stimuli.

This hypothesis of differential susceptibility to the action of thiocarbonamides on a genetic basis at least allows a fresh approach to be made to the aetiological problems of thyroid disease and is capable of proof both by laboratory and family studies.

[§] See also Clements, p. 133 of this number of the Bulletin.—Ed.

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THYROID AUTO-IMMUNITY

I. M. ROITT M.A. D.Phil.

Courtauld Institute of Biochemistry
Middlesex Hospital Medical School, London

DEBORAH DONIACH M.D.

Institute of Clinical Research
Middlesex Hospital Medical School, London

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References

Investigations on the abnormalities of serum proteins in thyroid diseases (Fromm, Lascano, Bur & Escalante, 1953; Luxton & Cooke, 1956; Skillern, Crile, McCullagh, Hazard, Lewis & Brown, 1956; Roitt, Doniach & Campbell, 1956; Doniach & Hudson, 1957; Greene, Morgan & Bird, 1958) led to the finding of thyroid auto-antibodies in Hashimoto's disease (Roitt, Doniach, Campbell & Hudson, 1956; Witebsky, Rose, Terplan, Paine & Egan, 1957). Since then the subject has been intensively investigated in a number of centres, and patients with various thyroid diseases have been surveyed for the presence of circulating thyroid antibodies. The results have been summarized in recent reviews (Owen, 1958; Mahaux, 1959; Doniach, Roitt & Hudson, 1959a).

The histological parallel between human auto-immune thyroiditis and the experimental lesions obtained by immunizing animals with homologous thyroid extracts, described by Rose & Witebsky (1956), led to the suggestion that the process underlying the disease in man was directly related to the presence of the circulating antibodies (Doniach & Roitt, 1957). The first simple hypothesis in which a chain reaction was postulated between these antibodies and thyroid acini is not consistent with more recent experimental findings, and it now appears that the antibodies identified serologically are not the direct cytotoxic factors. Attempts to produce thyroiditis in monkeys by using sera from patients with Hashimoto's disease (Hashimoto serum) have so far been unsuccessful, but recent experiments show that these sera give rise to cytotoxic effects when added to thyroid cells in tissue culture (Pulvertaft, Doniach, Roitt & Hudson, 1959), owing to the presence of a separate factor.

Two of the thyroid auto-antigens have been investigated in some detail; studies with Coons' fluorescent antibody technique[1] have been made on their histological localization, and their subcellular distribution has been studied in tissue-fractionation experiments.

The use of the serological tests on patients followed up over a period of three years has focused attention on a form of auto-immune thyroiditis with a subacute onset and has emphasized its distinctness from de Quervain's giant-cell thyroiditis now known to be caused by a virus (Eylan, Zmucky & Sheba, 1957; Felix-Davies, 1958).

Milder and earlier forms of lymphadenoid goitre can now be diagnosed, and their clinical and laboratory features differ in certain respects from the more advanced classical forms of the disease. Adult primary myxoedema appears to be a variant of auto-immune thyroiditis and it has been suggested that some cases of congenital athyreotic cretinism may result from placental transmission of antibodies and/or cytotoxic factors from the mother (Beierwaltes, Dodson & Wheeler, 1959; Blizzard, R. M., Chandler, R. W., Landing, B. H., Sutherland, J. M., Esselborn, V. M., Hamwi, G. J., Petit, M., Guest, G. M. & Skillman, T. G., communication to the Annual Meeting of the American Goiter Association, 1959).

1. Detection of Thyroid Auto-Antibodies

a. Circulating Antibodies

The principal auto-antigens so far known to be involved in Hashimoto's disease, and the methods used to detect their antibodies, are given in Table I. Precipitating antibodies to thyroglobulin are most conveniently demonstrated by allowing the serum to react with either a saline extract of thyroid or a purified thyroglobulin preparation in agar gel, using the Oudin or Ouchterlony techniques (Doniach & Roitt, 1957; Roitt & Doniach, 1957). Where the antibody level is insufficiently high to give a precipitin reaction, the more sensitive tanned red cell agglutination (TRC) technique of Boyden (1951) may be employed. Red cells, following treatment with tannic acid, may be coated with thyroglobulin and are then agglutinable by the appropriate antibodies. Titres of up to five million have been obtained with sera from cases of chronic thyroiditis, clearly showing the high sensitivity of the method. It has been possible to prepare formolized antigen-coated red cells which are stable for many months in the refrigerator and which are

[1] See Belyavin, *Brit. med. Bull.* 1959, 15, 193.—Ed.

TABLE I. The Auto-Antigens Implicated in Hashimoto's Disease, and their Antibodies

Auto-antigen	Detection of auto-antibodies
Thyroglobulin (stored in colloid)	Precipitation Tanned red cell agglutination Radioactive co-precipitation Coons' fluorescent antibody technique on fixed sections Passive cutaneous anaphylaxis
Intracellular (microsomal) antigen	Complement fixation Coons' fluorescent antibody technique on unfixed sections

simple to use for clinical tests (Fulthorpe, Roitt, Doniach & Couchman, unpublished work, 1959). Ager, Hutt & Smith (1959) recently devised a similar test for thyroglobulin antibodies, utilizing bentonite particles coated with the antigen.

For quantitative studies, classical precipitin curves can be made, but for low-titre sera or non-precipitating antibodies a radioactive co-precipitation method may be used (Roitt & Doniach, 1959). The serum is treated with an excess of [^{131}I]thyroglobulin, and the immune complexes formed are co-precipitated with rabbit anti-human γ-globulin. The radioactivity of the washed precipitate provides a measure of the thyroglobulin antibody in the Hashimoto serum.

With certain rare exceptions (Goudie, Anderson & Gray, 1959c; Belyavin & Trotter, 1959), Hashimoto sera do not deviate complement when reacting with thyroglobulin. However, most patients with Hashimoto's disease have distinct antibodies which fix complement with extracts of thyrotoxic thyroid gland (Trotter, Belyavin & Waddams, 1957) and are directed against a "microsomal" antigen. The complement-fixation (CF) test is best carried out in Perspex agglutination trays by the semi-micromethod of Donnelly (1951). Sera are screened at 1 in 4 and 1 in 20 dilutions, and positive sera are then titrated in serial dilutions up to 512, using 2 minimum haemolytic doses of complement for the test. It is important to combine TRC and CF tests in all patients suspected of Hashimoto's disease, since the respective thyroglobulin and microsome antibodies can be present together or separately in individual cases.

b. The Cytotoxic Factor of Serum from Patients with Hashimoto's Disease

Hashimoto serum has a cytotoxic effect on trypsinized human thyroid cells in tissue culture (Pulvertaft *et al.* 1959). Inactivation at 56° C. for 30 minutes abolishes the activity, but sera freshly stored at −20° C. for three months were still cytotoxic. Cells set up in primary culture in the presence of Hashimoto serum fail to grow, while cells cultured for 18–24 hours in normal serum and then transferred to the Hashimoto serum are killed. However, after 36 hours' growth the thyroid cells are no longer affected by subsequent exposure to the cytotoxic serum.

Cytotoxic effects have been obtained with Hashimoto sera in which the routine tests for thyroglobulin or for CF antibodies were negative, suggesting that the cytotoxic factor is distinct from these antibodies. The nature of the cytotoxic factor, and its relationship to the development of progressive thyroiditis as opposed to mild focal auto-immune lesions, has not yet been elucidated.

c. Skin Sensitivity and Passive Cutaneous Anaphylaxis

Intradermal injection of human thyroid extract into patients with Hashimoto's disease has been shown to produce a reaction, with erythema and induration reaching a maximum at 24 hours and usually obtained in patients with precipitins (Buchanan, Anderson, Goudie & Gray, 1958; Witebsky, 1959). It is not yet clear whether these cutaneous reactions should be interpreted as Arthus phenomena or as manifestations of "tuberculin type" hypersensitivity, since a late appearance of maximum response has also been observed in severe Arthus reactions. Skin tests with thyroid extracts in auto-immunized animals (Rose & Witebsky, 1959a) have been considered to be of delayed type.

Passive cutaneous anaphylaxis has been obtained in guinea-pigs by use of thyroglobulin and precipitating Hashimoto sera (Ovary, Randall, Witebsky, Rose, Shulman & Metzgar, 1958), but not with sera showing only complement-fixing (CF) antibodies.

Blizzard, Hamwi, Skillman & Wheller (1959) have noted that penicillin-sensitivity is more frequent in patients with Hashimoto's disease, and Beare (1958) has found an increased incidence of allergic manifestations in a group of such patients.

2. Thyroid Auto-Antigens

a. Thyroglobulin

Evidence from Ouchterlony gel-plate studies and from immunoelectrophoresis strongly implicated thyroglobulin as the antigen responsible for the precipitin reaction given by Hashimoto sera with crude thyroid extracts (Roitt, Campbell & Doniach, 1958). This was confirmed by the finding that 96% of the radioactivity of a thyroglobulin preparation isolated from the gland of a patient given ^{131}I could be precipitated by a Hashimoto serum.

Thyroglobulin antibodies may give quantitative precipitin curves of the "rabbit" type (White, 1957) or of the "horse-flocculation" type with the formation of soluble complexes in antibody excess (Roitt & Doniach, 1959).[3] The antibodies may be of the "7S" or "19S" type (Pressman, James, Yagi, Hiramoto, Woernley & Maxwell, 1957; Korngold & van Leeuwen, 1959), while some patients have antibodies in 7S and macroglobulin fractions (Fahey & Goodman, unpublished, 1959). The sedimentation characteristics in the ultracentrifuge have not yet been correlated with the type of precipitin curve. In some instances the precipitin test in agar shows a clear zone instead of—or in addition to—an opaque line and probably reflects the formation of non-aggregating antigen–antibody complexes (Goudie, Anderson & Gray, 1959a). Although the Coons technique has shown that Hashimoto serum can react with the patient's own gland, a case has now been described in which the precipitin reaction could be obtained only with other thyroid glands, although the patient's own gland reacted with other Hashimoto sera (Anderson, Goudie & Gray, 1960). Similar phenomena have been reported in pancreatitis (Thal, Murray & Egner, 1959) and in lupoid hepatitis (Mackay & Larkin, 1958), and may be interpreted on the basis of immunization to only a proportion of the available antigenic determinants and selective blocking of these by antibody.

In gel-plate diffusion systems, certain Hashimoto sera give two precipitation lines with thyroglobulin even when present in equivalent proportions. Double-line patterns can merge with the single line given by many Hashimoto sera or can give rise to "spur" formation with other sera. This probably implies the existence of two molecular species in thyroglobulin, having certain antigenic determinants in common (Korngold & van Leeuwen, 1959).

Evidence has been obtained for the presence of a colloid auto-antigen other than thyroglobulin. In certain patients with Hashimoto's disease, who have raised flocculation tests and high γ-globulins, but no thyroglobulin or CF microsome antibodies, the colloid of thyroid sections stained by the Coons technique gives a pattern distinct from that obtained with thyroglobulin precipitins (Balfour, Doniach & Roitt, un-

[3] In the "rabbit precipitin" type of curve the antigen is completely precipitated when the antibody is in excess; in the "horse-flocculation" type the antigen–antibody complex is soluble in the presence of excess of antibody.—Ed.

published work, 1959). The presence in these sera of incomplete antibodies to thyroglobulin was excluded by co-precipitation studies with [^{131}I]thyroglobulin and anti-γ-globulin serum, and by tests for blocking antibody.

b. Complement-Fixing Antigen

Some patients have antibodies directed only against the CF antigen which is present in high concentration in the thyrotoxic gland, but have no thyroglobulin antibodies, and vice versa. Further, removal of the precipitins from a Hashimoto serum by flocculation with thyroglobulin does not affect the CF titre. Thus the CF antigen behaves independently of the thyroglobulin immune system and this is further borne out by studies on the tissue localization of the antigens by immunohistochemical methods and by fractionation studies on thyroid gland homogenates by differential centrifugation.

Localization of antigens. The Coons fluorescent antibody technique has been applied to the study of thyroiditis by several groups of workers. White (1957, 1959) showed that a fluorescein-conjugated Hashimoto serum reacted not only with a normal thyroid gland but also with the patient's own gland, giving specific staining in the colloid and in scattered round globules within the epithelial cells; extensive extravasation of thyroglobulin from injured follicles was also observed. Colloid staining was similarly obtained when animal thyroids were treated with experimentally produced auto-antibodies (Beutner, Witebsky, Rose & Gerbasi, 1958). Witebsky's group have further demonstrated cross-reactions of the human thyroid antibodies with rabbit and dog colloid (Beutner, Witebsky & Gerbasi, 1959). Hiramoto, Engel & Pressman (1958) also obtained colloid staining, using conjugates prepared with tetramethyl rhodamine isocyanate. Cytoplasmic staining of the thyroid epithelial cells was reported by Landing, West & Esselborn (1958) with some sera from patients with chronic thyroiditis, using unfixed thyroid sections, but the results were not related to serological findings. It has since been demonstrated that diffuse cytoplasmic staining corresponds with the presence of the CF microsome antibody (Holborow, Brown, Roitt & Doniach, 1959). This staining can be inhibited by prior absorption of the sera with thyroid microsomes but is unaffected by treatment with thyroglobulin. Failure to observe cytoplasmic staining in ethanol-fixed sections is due to inactivation of the microsomal antigen and is paralleled by loss of antigenic potency in the CF test, with microsome fractions treated with ethanol. Thyroglobulin staining, on the other hand, can be obtained only in sections fixed with ethanol or formalin, since colloid is leached out from unfixed tissue. These studies further confirm the separate nature of these two immune systems.

Tissue-fractionation studies. The distribution of CF antigen was studied in fractions obtained from thyroid gland homogenates by differential centrifugation according to the scheme devised by Schneider & Hogeboom (1950). Approximately 90% of the CF activity found in the subcellular fractions was concentrated in the microsome pellet (Roitt & Doniach, 1958; Belyavin & Trotter, 1959). In contrast, the bulk of the thyroglobulin was recovered in the supernatant fraction. CF activity was obtained with microsomal fractions from normal thyroids and non-toxic goitres but was far weaker than that found in thyrotoxic glands. This difference between the antigenic potencies of thyrotoxic and non-toxic colloid goitres is illustrated in Table II. The relative concentration of antigen in homogenates of the gland was determined by a quantitative CF test (Roitt, Doniach & Couchman, 1960). The far higher levels of antigen in the hyperplastic glands expressed as arbitrary units per gram of wet tissue are not merely a reflection of their greater cellularity, since comparable differences may be seen when the activities are expressed as units per mg. deoxyribonucleic acid, which is a measure of the concentration per cell; neither could these results be attributed simply to differences in the levels of microsomal protein or ribonucleic acid. It would appear that the antigenic potency of thyroid cells is related more closely to their functional activity. Evidence in support of this view has been obtained from a study of patients with a hyperfunctioning adenoma in one lobe of the thyroid. The functional activity of the normal lobe is suppressed, since overactivity of the adenoma leads to inhibition of the secretion of thyrotrophic hormone (TSH) by the pituitary. In these cases the CF activity of the adenoma was several times higher than that of the normal lobe (Table III). Anderson, Goudie & Gray (1959) have found high CF activity in the hyperplastic thyroids of goitrous cretins with various forms of dyshormonogenesis due to enzyme deficiencies; this implies that the antigen is not necessarily related to effective hormone production.

TABLE II. Complement-Fixing (CF) Microsomal Antigen in Thyrotoxic and Non-Toxic Thyroid Glands

Patient			Diagnosis	CF microsomal auto-antigen*			
Initials	Sex	Age (years)		Units/g. thyroid	Units/mg. deoxyribonucleic acid	Units/mg. microsomal protein	Units/mg. microsomal ribonucleic acid
F.S.	Male	58	Thyrotoxicosis	6,200	3,000	620	15,500
N.K.	Male	27	Thyrotoxicosis	3,900	1,220	260	7,800
M.B.	Female	31	Thyrotoxicosis	2,700	1,220	220	2,200
C.P.	Female	43	Thyrotoxicosis	1,700	660	118	1,300
W.K.	Female	38	Non-toxic colloid goitre	400	220	40	1,100
F.C.	Female	61	Non-toxic colloid goitre	320	170	—	—
B.T.	Female	30	Non-toxic colloid goitre	150	104	16	340

* 1 unit of antigen gives 50% lysis in the complement-fixation test with a standard Hashimoto serum in the presence of three 50% haemolytic units of complement.

TABLE III. Complement-Fixing (CF) Microsomal Antigen in Patients with Single Functioning Adenoma

Patient			CF microsomal antigen* (units/g. thyroid tissue)	
Initials	Sex	Age (years)	Adenoma	Normal lobe
E.K.	Female	14	880	65
M.H.	Female	74	2,200	100
K.G.	Female	49	510	120

* Units as in Table II

3. Experimental Auto-Immune Thyroiditis

The production of circulating thyroid auto-antibodies in rabbits by immunization with rabbit thyroid extracts combined with Freund's adjuvant was first reported by Witebsky & Rose (1956) (also Witebsky, Rose & Shulman, 1956). Histological examination of the thyroid glands of these animals after 2–3 months revealed varying degrees of infiltration by lymphoid cells and eosinophils around thyroid follicles which in extreme cases contained little or no colloid (Rose & Witebsky, 1956). The over-all appearance resembles that seen in the Hashimoto goitre, although nothing comparable to the eosinophilic Askanazy cell change of the thyroid epithelium has been observed in the rabbit glands and no goitres resulted. Witebsky and his colleagues (Witebsky *et al.* 1957) were able to reproduce the pathological effects when purified thyroglobulin preparations were substituted for the crude extracts; they further showed that similar changes could be induced in guinea-pigs and dogs. These investigators have studied the cross-reactions of the auto-antibodies with thyroid antigens of other species (Rose & Witebsky, 1959b) and were able to observe thyroiditis in animals immunized with cross-reacting heterologous thyroid antigens (Witebsky & Rose, 1959).

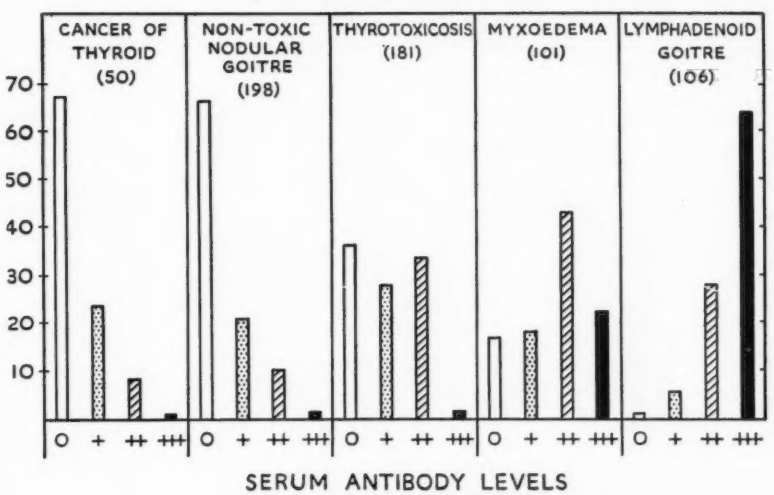
To study the mechanisms underlying the experimental thyroid lesion, it was felt desirable to produce severe lesions with regularity in an animal species of relatively uniform genetic make-up, readily available and conveniently handled in large numbers. Using a closed colony of rats derived from a Wistar strain, it was possible to evoke a marked thyroiditis in over 90% of animals, 3–4 weeks after injections of rat thyroid extract or thyroglobulin in a slightly modified Freund's adjuvant (Jones & Roitt, unpublished work, 1959). Of particular interest in relation to the pathogenesis of human thyroiditis is the finding that the lesion present at four weeks in the rat thyroid, as confirmed by hemithyroidectomy, generally undergoes regression if no further immunizing injections are given; total regression of moderately severe thyroiditis has been observed 1–2 months after the hemithyroidectomy. Another feature seen in some animals is involvement of the thyroid capsule and adjacent tissue by the inflammatory process, leading to a fusion of the gland with the trachea, oesophagus and pretracheal muscles, reminiscent of that seen in Riedel's struma but differing from it by the absence of dense fibrous tissue.

4. Clinical Aspects

a. Diagnostic Significance of Serological Tests

Figure 1 shows the incidence of auto-antibodies detected by the precipitin, TRC and CF tests in thyroid diseases. Titres were classed as low, medium and high by comparison with those obtained in untreated Hashimoto's disease, which gave 97% positive results in our series when both immune systems are considered together. In primary myxoedema antibodies were found in 83% of cases, while the corresponding figures in thyrotoxicosis, non-toxic colloid goitre and thyroid cancer were 63%, 33% and 32% respectively. Other published clinical surveys include those of Paine, Terplan, Rose, Witebsky & Egan (1957), Goudie, Anderson and their co-workers (Goudie, Anderson, Gray, Clark, Murray & McNichol, 1957;

FIG. 1. Incidence of Circulating Thyroid Auto-Antibodies in Thyroid Diseases



Doniach, Hudson & Roitt (1960) by permission of British Medical Journal

Ordinates: percentage of patients

The incidence was estimated from the precipitin reaction in agar, tanned red cell agglutination (TRC) and complement-fixation (CF) test. The levels of serum antibodies were divided into weak, medium and high titres as follows:

Weak (+): TRC titres 5–250 and/or CF titres less than $\frac{1}{10}$ serum dilution, using two minimum haemolytic doses of complement for the test

Medium (++): TRC titres 2,500–25,000 and/or CF titres of 20 or over

High (+++): positive precipitins; in these cases the TRC titre was between 25,000 and over 2,500,000 (serum dilutions in steps of 10 made with a single pipette), while complement-fixing antibodies were usually present.

Goudie, Anderson & Gray, 1959a, 1959b, 1959c), Owen & Smart (1958), Belyavin & Trotter (1959), Blizzard *et al.* (1959), Cline, Selenkow & Brooke (1959), Hubble (1959) and Cervino, Paseyro, Grosso & Estable (1959). The results show reasonable agreement, considering the variations in the methods and choice of material. By far the highest titres are found in Hashimoto patients (cf. Mahaux & Pirart, 1959), but comparable levels are found in 20% of cases of primary myxoedema, while titres in other thyroid diseases are generally far lower. Only four cases of true invasive fibrous thyroiditis (Riedel's) have been encountered in our series, and all had low antibody levels. Thyroid antibodies of low titre are found in a small proportion of middle-aged women in the absence of overt thyroid diseases, and this has been correlated to a certain extent with lymphadenoid foci in the gland (Goudie *et al.* 1959b).

Auto-antibody levels in carcinoma of the thyroid are sufficiently low to make the tests a useful adjunct in the differential diagnosis between Hashimoto's disease and thyroid malignancy (Doniach, Roitt & Hudson, 1958). The presence of precipitins is indicative of active and wide-spread lymphadenoid change and this is the safest test to rely on. In the rare cases where a cancer co-exists with lymphadenoid goitre, precipitins may be demonstrable (Stuart & Allan, 1958a) and therefore it is important, besides using other diagnostic criteria, to observe the response of the goitre to thyroxine and to explore surgically all suspicious cases. Conversely, the antibody tests may be negative or of low titre in some early Hashimoto goitres or in rapidly progressing ones. The diagnostic value of the immunological tests is considerably enhanced when they are combined with sero-flocculation reactions, since patients with low antibody titres may have raised γ-globulins, possibly owing to as yet undetected antibodies. On the other hand, patients may show appreciable precipitin levels and yet give normal results in the thymol and zinc sulphate turbidity tests (Doniach, Hudson & Roitt, 1957). Antibody levels usually fall after thyroidectomy, but in occasional cases antibodies have first appeared after operation (Witebsky, Rose & Shulman, 1958).

Thyroid antibody tests are helpful in diagnosing early or doubtful primary myxoedema and in predicting the likelihood of post-operative hypothyroidism in the occasional thyrotoxic patients who have fairly advanced lymphadenoid changes accompanied by high auto-antibody titres.

b. Atypical Forms of Lymphadenoid Goitre

Apart from classical Hashimoto's disease, which starts insidiously in middle-aged women and in which a firm, diffuse and symmetrical goitre develops and gives rise to varying degrees of hormonal deficiency, some variants of auto-immune thyroiditis have been observed since the application of antibody tests (Doniach, Hudson & Roitt, 1960).

Lymphadenoid goitre with subacute onset. Patients in this group present with a 4–8 weeks' history of neck swelling with discomfort over the thyroid area, sometimes amounting to pain which radiates to the ears or back of head. Fever was present in a proportion of cases (Druez, Hainaut, Parmentier & Musin, 1958; Mogensen, 1958), and some patients have given a history of preceding upper respiratory infections. There is a diffuse, firm and sometimes tender goitre, and the differential diagnosis, on clinical grounds, from de Quervain's disease is not easy except for the lesser severity of the pain and constitutional symptoms in the former group. The condition differs from de Quervain's thyroiditis in that the iodine uptake is usually normal or raised and the thyroid auto-antibody titre is high at an early stage. In de Quervain's disease the iodine uptake is almost invariably suppressed at the height of the illness, and the thyroid antibodies are absent or found only in low titre. The basal metabolic rate (BMR) is usually normal in auto-immune thyroiditis of this type but, if the process is very active, myxoedema can supervene within 2–3 months and thyroid failure is then irreversible. The γ-globulins are raised, in contrast with the normal levels in de Quervain's disease in which the α_2-globulins are elevated owing to tissue breakdown (Stemmermann, 1956). The sedimentation rate and protein-bound iodine (PBI) may both be raised and do not differentiate the two conditions in the acute phase. After a variable interval of time, in cases of auto-immune thyroiditis starting subacutely, the goitre may become painless and it may take several years before the patient begins to show signs of incipient hypothyroidism, particularly if active regeneration is competing with the destructive process in the thyroid.

It is not known whether these subacute cases of auto-immune thyroiditis are initiated by a virus infection. The occasional history of a preceding upper respiratory infection may suggest a connexion, and a few cases of myxoedema appear to have followed mumps thyroiditis (e.g., Eyquem, Calmettes & Decourt, 1959). However, the high antibody titre found so soon after the apparent onset is difficult to explain on this basis. Possibly such cases have a pre-existing silent thyroiditis which is boosted into increased activity by a virus infection. The fact that de Quervain's disease does not as a rule lead to progressive auto-immunity suggests that virus thyroiditis itself is insufficient to initiate active lymphadenoid lesions and that other factors must be involved.

Auto-immune thyroiditis with silent or remitting course. Hormonal failure is not the inevitable outcome in auto-immune thyroiditis starting subacutely. In some cases the goitre and pain subside and the condition appears to enter a silent phase with persistent antibodies as the only abnormality. Repeated subacute attacks have been seen and it is not yet known whether these patients will ultimately become myxoedematous.

Auto-immune thyroiditis is probably often entirely silent for many years, as is seen from the number of patients with primary myxoedema who give no history of any preceding thyroid disorders. Since we have looked specifically for evidence of previous infections in cases of spontaneous myxoedema, a few patients have admitted having neck pain or a feeling of tightness in the thyroid region months or years before the onset of hypothyroidism. Such episodes may pass unnoticed or be diagnosed as tonsillitis or pharyngitis at the time but may represent the active onset of the lymphadenoid process.

c. Effect of Varying Antibody Pattern on Thyroid Function Tests in Lymphadenoid Goitres

Whereas 75% of patients with Hashimoto's disease have both thyroglobulin and microsome (CF) antibodies, about 10% have only CF antibodies, while 3–5% of patients give negative results by the three serological methods used routinely. Histologically the glands of these patients show acinar breakdown (Stuart & Allan, 1958b) and extensive leakage of colloid which can be demonstrated by the fluorescent antibody technique; furthermore Owen & McConahey (1956) have demonstrated the presence of a circulating thyroglobulin-like

protein in a proportion of patients with Hashimoto's disease. It is not known why thyroglobulin antibodies fail to develop in some cases, but absence of precipitins may modify the clinical picture and thyroid function tests, when thyroglobulin reaches the circulation in appreciable amounts and is not removed and inactivated by combining with antibody. The consequences of freely circulating thyroglobulin would be symptoms of mild hyperthyroidism or intolerance to administered thyroid hormone, a serum PBI higher than expected from the BMR and a responsiveness to injected TSH. All these features have been reported in a proportion of cases in the literature, particularly in young people with early forms of the disease (Gribetz, Talbot & Crawford, 1954). We have observed these characteristics in patients with high CF titres and no thyroglobulin antibodies, many of whom remained euthyroid for long periods without treatment. Absence of thyroglobulin antibodies is not confined to lymphadenoid goitres of short duration, and some patients become myxoedematous without showing precipitins; but at this stage the clinical picture and function tests are not influenced by the antibody pattern, for there is little colloid remaining in the gland.

d. Factors Predisposing to Thyroid Auto-Immunity

Graves's disease must predispose to the development of auto-immunization, since two-thirds of thyrotoxic patients have low-titre auto-antibodies. However, in the great majority of cases the lesions remain strictly focal, of low-grade activity, and are of no clinical significance. Thyrotoxicosis rarely merges into Hashimoto's disease, but if one takes into account the patients who become myxoedematous many years after a thyrotoxic episode and those who have post-thyroidectomy myxoedema as a result of pre-existing lymphadenoid replacement in the gland, the auto-immune process would appear to lead to substantial thyroid destruction in about 10% of thyrotoxic patients. However, there are patients with fairly wide-spread lymphadenoid lesions who remain euthyroid and even develop recurrent thyrotoxicosis after removal of seven-eighths of their goitre. Such patients usually have persistently low antibody levels.

Although, in our series, Hashimoto's disease was 17 times more common in women than in men, in thyrotoxicosis the incidence of lymphadenoid lesions and of ultimate myxoedema is of equal frequency in men and women. Another interesting problem in this connexion is that of malignant exophthalmos which is found more frequently in men. An immunological survey in this type of case revealed a large proportion of high-titre sera among the patients with progressive exophthalmos. In certain cases of malignant exophthalmos the extra-ocular muscles are invaded by lymphocytes, and lesions resembling those of Sjögren's syndrome are observed in the lachrymal glands. It is therefore possible that the myositis may prove to be of auto-immune origin and be related to that described in systemic lupus erythematosus (Gold & Gowing, 1953).

Thyroid auto-antibodies are found at least three times more often in patients with colloid non-toxic goitres than in the general population of comparable age-groups, and their incidence is six times higher in thyrotoxic patients. Thus local abnormality in the thyroid gland appears to play an important role in the initiation of thyroid auto-immunity and, since all goitres are more common in women, this may partly account for the sex differences in Hashimoto's disease.

There is an increased incidence of thyroid disorders in the relatives of patients with Hashimoto's disease. The most common association is with thyrotoxicosis, usually in mother or sister; we have also seen several cases of primary myxoedema in siblings and some cases associated with a high incidence of familial colloid goitre. Dunning (1959) has reported three cases of Hashimoto's disease in one family. The coexistence of Hashimoto's thyroiditis with other diseases of suspected auto-immune aetiology has been reported. Lymphadenoid goitre may be associated with progressive hepatitis and nephrosis (Luxton, 1957; Doniach, Roitt & Hudson, 1959b), with acquired haemolytic anaemia (Wasastjerna, 1959), Addison's disease (Anderson *et al.* 1959) and collagen diseases (cf. Doniach & Roitt, 1959).

Although the occurrence of low-titre thyroid antibodies and of focal lesions is common in all types of goitre, the progressive replacement of the thyroid with lymphadenoid tissue which characterizes Hashimoto's disease is relatively rare. The part played by the cytotoxic factor and the various forms of immunological hypersensitivity (cf. Waksman, 1959) in the progression of the disease has yet to be evaluated.

ACKNOWLEDGEMENTS

We are grateful to Mr R. Vaughan Hudson for his close association with the work on thyroid auto-immunity from its inception. We thank Professor Sir Charles Dodds and Professor F. Dickens for their unfailing support and Professor A. Kekwick and the Clinical Research Committee for facilities afforded in the Institute of Clinical Research. We are greatly indebted to Mr K. Couchman for skilled assistance. The work is supported in part by a grant from the British Empire Cancer Campaign to the Middlesex Hospital Medical School and by the Will Edmonds Fund of the Royal College of Physicians. Some equipment was generously donated by the Mary Kinross Charity Fund.

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THE PASSAGE OF THYROID HORMONE FROM MOTHER TO FOETUS AND ITS RELATION TO FOETAL DEVELOPMENT

C. OSORIO M.D.*

N. B. MYANT D.M. B.Sc.

Medical Research Council
Experimental Radiopathology Research Unit
Hammersmith Hospital, London

1 Passage of thyroid hormone from mother to foetus
2 Role of binding substances in distribution of thyroid hormone between mother and foetus
3 Passage of thyroid hormone from foetus to mother
4 Requirement of the foetus for thyroid hormone
5 Function of the foetal thyroid
6 Conclusion
References

1. Passage of Thyroid Hormone from Mother to Foetus

If normal foetal development requires thyroid hormone, this could reach the foetus either from the foetal thyroid gland or from the mother. Early attempts to discover how far the mother's hormone is available to the foetus led to conflicting results. Courrier & Aron (1929) could find no change in the appearance of the thyroid of new-born dogs when the mother was injected with thyroid hormone during pregnancy. Arguing that if the injected thyroxine had reached the foetus it would have caused hypoplasia of the foetal thyroid by inhibition of the foetal pituitary, they concluded that thyroxine does not cross the placenta. However, they gave no evidence that the foetal thyroid was controlled by the foetal pituitary at the period of gestation when the injections were made. Grumbrecht & Loeser (1938) observed flattening of the epithelial cells of the foetal thyroid in rats and guinea-pigs when the mother was given injections of thyroxine. They concluded that the thyroxine had crossed the placenta. Theresa (1939) thought that the thyroids of new-born rabbits were more differentiated than normal if the mother was injected with thyroid hormone from the 15th day of pregnancy until the end of gestation. This was taken as evidence that the injected hormone had reached the foetus, although no one else has confirmed the observation that an excess of thyroid hormone stimulates the foetal thyroid.

The experiments of Peterson & Young (1952), who worked with guinea-pigs, confirmed the suggestion that thyroid hormone does cross from the mother to the foetus. It was already known that treatment of a pregnant animal with thiouracil causes hyperplasia of the foetal thyroid, in addition to that of the maternal thyroid, and that the hyperplasia is due indirectly to release of thyrotrophic (thyroid-stimulating) hormone (TSH) brought about by the lowered blood thyroxine concentration. But it was not known whether the effect on the foetal thyroid is due to stimulation by TSH secreted by the mother or by the foetus. Peterson & Young (1952) showed that TSH cannot reach the foetus from the mother. They therefore concluded that the hyperplasia of the foetal thyroid must be due to secretion of TSH by the foetal pituitary. They then showed that injections of thyroxine into pregnant animals treated with propyl thiouracil markedly diminished the hyperplasia of the foetal thyroid. Since the inhibitory action of thyroxine on the secretion of TSH by the pituitary is very specific, they were justified in concluding that the thyroxine given to the mother had reached the foetal pituitary. In an analogous experiment, Knobil (1958) has shown that tri-iodothyronine and thyroxine cross the placenta in rats. Postel (1957), on the other hand, failed to observe any reduction in the weights of perchlorate-induced goitres in guinea-pig foetuses when he injected the mother with tri-iodothyronine throughout the last stages of pregnancy. He concluded that tri-iodothyronine does not cross the guinea-pig's placenta in physiological amounts.

Although this approach is capable of showing whether or not the thyroid hormone crosses the placenta, it does not give quantitative information, especially with regard to the rate of passage of hormone from mother to foetus and the period of gestation at which it occurs. The only valid conclusion from the experiments of Peterson & Young (1952), who gave repeated injections of thyroxine to the mother from the 38th until the last day of pregnancy, is that the thyroxine reached the foetus at some time during this period. More exact quantitative information can be obtained by using labelled thyroxine, though this method also has its limitations, as will become apparent below.

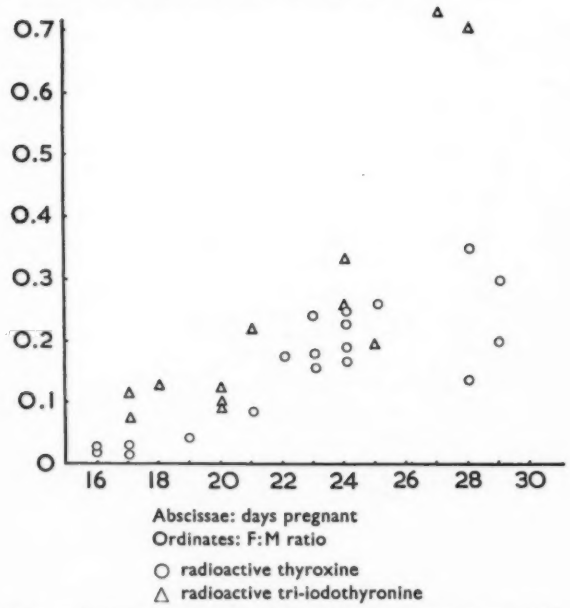
Hirvonen & Lybeck (1956) and Lybeck (1956) injected radioactive thyroxine and tri-iodothyronine into guinea-pigs during late pregnancy and measured the concentration of radioactivity in the serum 2–4½ hours later. The concentration in the serum of the foetus was much lower than that in the mother's serum; in some cases no activity could be detected in the foetal serum when it was easily detectable in the maternal serum. These results suggest that the passage of hormone across the guinea-pig's placenta is very slow. This is not necessarily at variance with the findings of Peterson & Young (1952). Hirvonen & Lybeck (1956) gave no evidence as to how nearly equilibration between maternal and foetal hormone had been achieved during the time between the injection and the blood sampling. Even if the rate at which hormone is transferred from mother to foetus is very slow, considerable amounts could reach the foetus if a period of many days were allowed, as in the experiments of Peterson & Young (1952). It is also possible that in guinea-pigs conditions become more favourable for the passage of hormone across the placenta at the end of pregnancy; this seems to be the case in rabbits and man (see below). The last few days of pregnancy must have been included in the experiments of Peterson & Young (1952), but may not have been in those of Hirvonen & Lybeck (1956), who did not say how old their foetuses were.

Nataf, Sfez, Michel & Roche (1956) claim that after an injection of radioactive tri-iodothyronine into pregnant rats the concentration of radioactive tri-iodothyronine in the foetal serum rises to nearly half that in the maternal serum within one hour, and that equilibration is nearly complete within two hours. If true, this indicates that the permeability of the rat's placenta to tri-iodothyronine is much greater than that of guinea-pigs, rabbits or man.

* In receipt of a Research Fellowship from The Wellcome Trust.

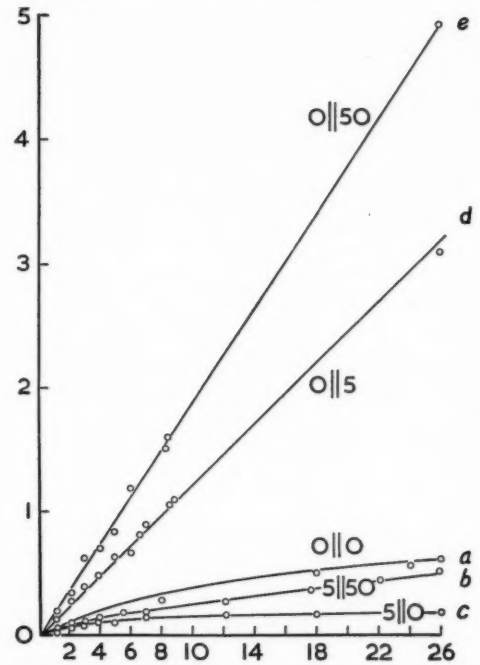
Hall & Myant (1956) and Myant (1958) have made observations on rabbits similar to those of Hirvonen & Lybeck (1956) on guinea-pigs. If radioactive thyroxine is injected into rabbits during the early stages of pregnancy, very little radioactivity is detectable in the foetal serum even if the samples are taken at 24 hours after the injection. If, however, the injection is made later in pregnancy, the concentration of radioactive thyroxine in the foetal serum at 24 hours rises to about a third of that in the maternal serum. When radioactive tri-iodothyronine is injected, the results are more or less the same, but at a given stage of pregnancy the foetal : maternal serum concentration ratio (F : M ratio) is higher for tri-iodothyronine than for thyroxine. Figure 1 shows the F : M ratio in a group of pregnant rabbits 24 hours after injections of radioactive thyroxine or tri-iodothyronine between the 17th and 30th days. The high concentration of radioactive hormone appearing in the foetal serum in late pregnancy might be thought to be due to foetal synthesis and secretion of radioactive hormone from radioiodide derived from the breakdown of the radioactive hormone in the mother. But this can be excluded, since the same results are obtained when the foetal thyroid is removed by decapitation of the foetus in utero before the injection is made. It could also be argued that hormone passes from the mother to the foetus equally freely at all stages of pregnancy, but that in early pregnancy the hormone reaching the foetus is rapidly de-iodinated or stored in the foetal organs. Hall & Myant (1956) have shown that neither de-iodination nor storage occurs to any extent in the foetus. It therefore seems most reasonable to interpret the results illustrated in fig. 1 by supposing that conditions become more favourable for the passage of hormone across the placenta as pregnancy advances.

FIG. 1. Foetal : Maternal Serum Concentration Ratio (F:M Ratio) of Radioactive Thyroxine and Radioactive Tri-Iodothyronine



Ratio at 24 hours after intravenous injection into pregnant rabbits at different stages of pregnancy. All values shown at time of injection.

FIG. 2. Equilibrium Dialysis of Solutions of Radioactive Thyroxine between Two Compartments



Abscissae: hours after adding radioactive thyroxine to left side
Ordinates: ratio of concentrations of radiothyroxine in right compartment to that in left compartment after adding radiothyroxine (1 μg.)

Each compartment held 15 ml. of solution, and was separated by a cellophan membrane (4 × 5 cm.). Constant rate of stirring maintained throughout dialysis. Temperature: +5° C.; pH of buffer: 8.6
Percentage of human serum (v/v) in fluid on left and right of membrane shown by values to left and right of parallel vertical lines; e.g., in *a*, both sides contained veronal buffer with no serum; in *b*, left side contained 5% serum, right side contained 50% serum.

In human beings, the passage of hormone from mother to foetus seems to be much the same as in rabbits. Radioactive thyroxine injected into the mother during the last few days of pregnancy reaches a relatively high concentration in the serum of the foetus at birth (Grumbach & Werner, 1956), but very little activity reaches the foetus if the injection is made early in pregnancy (Myant, 1958). As with rabbits, tri-iodothyronine passes from the mother to the foetus more readily than thyroxine at all stages of pregnancy. The clinical observations of Carr, Beierwaltes, Raman, Dodson, Tanton, Betts & Stambaugh (1959) also suggest that when a pregnant woman is given repeated doses of thyroxine some of it reaches the foetus.

2. Role of Binding Substances in Distribution of Thyroid Hormone between Mother and Foetus

An increase in the passage of hormone across the placenta could come about in several ways. It could be due to an

increase in the permeability of the tissues separating the mother from the foetus, or to an increase in the surface area of the placental blood vessels, or, if blood flow limits the rate of passage of hormone across the placenta, to an increase in blood flow through the placenta. The changes undergone by the rabbit's placenta during its development suggest that all these factors contribute. However, there is yet another which may be as important as any of the changes taking place within the placenta.

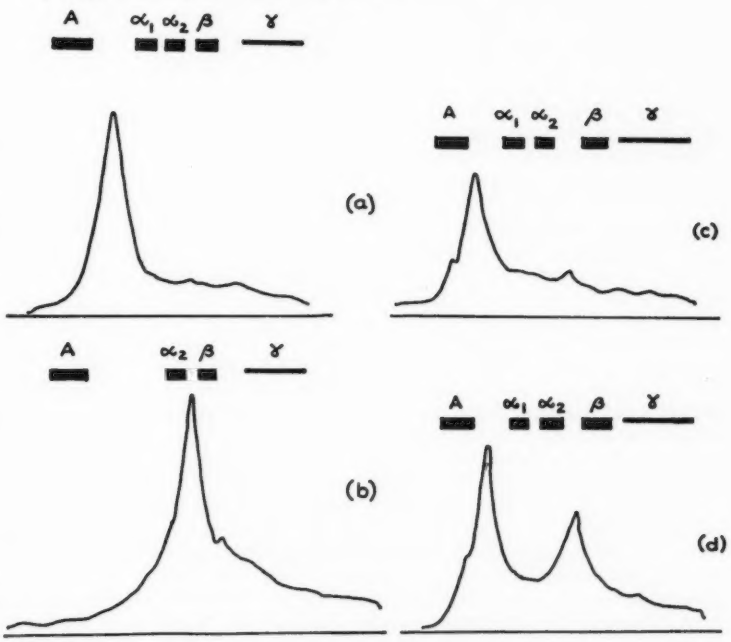
The thyroid hormone in the blood is reversibly adsorbed to a substance, generally assumed to be a protein, known as thyroxine-binding protein (TBP). In human beings, the affinity of TBP for thyroxine is such that under physiological conditions the concentration of free thyroxine is certainly less than 0.001 of the concentration of bound thyroxine, and may be much less than this (Robbins & Rall, 1957). The fact that the circulating hormone is bound to TBP must influence its passage from the mother to the foetus. For one thing, if the placenta is impermeable to TBP, only a very small proportion of the circulating hormone will be available for exchange across the placenta at any instant. Further, if the placenta is impermeable to TBP, but permeable to free thyroxine, the concentrations of total hormone in the maternal and foetal circulations at equilibrium will be in the ratio of their respective thyroxine-binding powers—"binding power" is used here, for want of a better term, to mean the product of binding affinity and binding capacity, as discussed by Myant & Osorio (1959).

A dialysis experiment will illustrate these points. Two fluid compartments of equal volume are separated by a cellophan membrane. Radioactive thyroxine is added to the left side, and the rate at which the concentration of radioactivity changes on the two sides is then measured. If both sides contain veronal buffer, the thyroxine distributes itself equally between the two compartments, with a half-period of about 13 hours (fig. 2, *a*). If some of the buffer on both sides is replaced by serum, the rate of equilibration across the membrane is much slower (fig. 2, *b*), presumably because only a small proportion of the thyroxine molecules is free to cross the membrane at any instant. If some of the buffer on the left side is replaced by serum, very little radioactive thyroxine appears on the right side (fig. 2, *c*). If, however, there is serum on the right side and only buffer on the left side, the concentration on the right side rises more quickly and to a higher value than if there is buffer on both sides (figs. 2, *d*; 2, *e*). Thus, although the membrane is moderately permeable to free thyroxine, as shown by fig. 2, *a*, the rate of equilibration and the ratio of the concentrations of total thyroxine on the two sides can be varied by altering the thyroxine-binding capacity of the fluid on either side of the membrane.

The possibility that the maternal and foetal circulations might be to some extent analogous to such a model led Osorio & Myant (1958) to examine the thyroxine-binding power of maternal and foetal rabbit serum at different stages of pregnancy. If a sample of serum containing radioactive thyroxine is submitted to paper electrophoresis, the thyroxine is carried away from the origin by the movement of the TBP to which it is adsorbed. The position of TBP in relation to the other serum proteins can then be found by measuring the distribution of radioactivity along the paper. In the serum of adult rabbits tested by electrophoresis in a veronal buffer, a sharp peak of radioactivity is seen between albumin and the α_1-globulin (fig. 3a). When the serum of a 25-day-old foetus is tested in the same way, the pattern of radioactivity is found to be quite different from that in maternal serum. All the activity is confined to a single peak between the α_2- and β-globulins (fig. 3b). There must, therefore, be two specific thyroxine-binding substances in rabbit serum with different electrophoretic mobilities: one in the foetus, which we have called foetal TBP, and the other in the adult, which we have called adult TBP.

Since adult and foetal TBP can be distinguished from each other, the partition of thyroid hormone between maternal and foetal serum can be studied in mixtures of the two. If a mixture of equal parts of foetal and maternal serum is tested after

FIG. 3. Distribution of Radioactivity along Paper Strip after Electrophoresis of Serum to which Radioactive Thyroxine (5 μg./100 ml.) Had Been Added



a: maternal serum from pregnant rabbit at 25th day
b: foetal serum from the same rabbit
c: mixture of equal parts of maternal and foetal serum at 19th day
d: mixture of equal parts of maternal and foetal serum at 25th day
Position of serum proteins is shown above each curve.
A: albumin; α_1, α_2, β, γ: globulin
Note that α_1-globulin is not present in foetal serum at 25th day.
Buffer: veronal at pH 8.6 and ionic strength 0.05

the addition of radioactive thyroxine, two peaks of radioactivity are found, the larger one at the position of adult TBP and the smaller at the position of foetal TBP (fig. 3d). From the areas under the two peaks it is possible to determine the proportions of the added thyroxine bound respectively by maternal and foetal TBP.

The distribution of radioactivity is found to vary with the age of the foetus from which the foetal serum is taken. At the 19th day, nearly all the thyroxine is bound by the adult TBP in the mixture (fig. 3c), but as the age of the foetus increases there is a progressive rise in the proportion bound by foetal TBP. By the 25th day, the foetal TBP binds nearly a third of the thyroxine (fig. 3d); by the 28th day the foetal TBP may carry nearly as much as the adult TBP. It has been shown (Myant & Osorio, 1959) that these results can be explained by a change in the concentration of foetal TBP in foetal serum during the last stages of pregnancy. At the 19th day, the concentration is very low: thereafter, it rises to a level such that the foetal serum is able to compete effectively in vitro for thyroxine in the presence of maternal serum.

The rise in the binding power of foetal serum would be expected to lead to an apparent increase in placental permeability to thyroid hormone, as observed in vivo. Further, if the passage of hormone across the placenta is influenced by the presence of TBP, this would explain why tri-iodothyronine reaches the foetus more readily than thyroxine, since it is not as firmly bound to TBP as thyroxine. However, without more evidence it cannot be assumed that the changes observed during late pregnancy are explicable solely in terms of the relative binding powers of the blood on either side of the placental barrier. When the partition of thyroxine between maternal and foetal serum is studied in vitro, complete equilibration is allowed to take place. However, it is not certain that equilibration across the placenta is achieved in vivo within 24 hours of an injection into the mother. It is impossible, therefore, to say how far the change in the F : M ratio (see fig. 1) is due to the rise in the concentration of foetal TBP, and how far to a change in the rate of equilibration between mother and foetus brought about by the factors mentioned above. In this connexion, it would be interesting to know whether the low F : M ratio in early pregnancy can be raised by artificially increasing the binding power of the foetal serum. One other possibility, for which Myant & Osorio (1960) have brought forward indirect evidence, is that the placenta becomes permeable to adult TBP at the end of pregnancy. This would facilitate the passage of thyroid hormone from the mother to the foetus.

The binding affinity of foetal TBP for thyroxine is several times greater than that of adult TBP (Myant & Osorio, 1959). The reason why foetal TBP does not acquire the larger share of the thyroxine in a mixture of maternal and foetal serum is that its binding capacity is much less than that of adult TBP, even at the end of pregnancy. (As discussed elsewhere (Myant & Osorio, 1959), binding capacity may be defined as the total number of binding sites in unit volume of serum, and is, therefore, directly proportional to concentration.) The high binding affinity of foetal TBP has certain consequences worth mentioning. When the binding of thyroxine is tested in a mixture of maternal and foetal serum in the presence of increasing concentrations of thyroxine, the proportion bound by foetal TBP falls as the concentration of thyroxine rises, since the binding sites on foetal TBP are saturated before those on maternal TBP. Conversely, the proportion bound by foetal TBP is greatest at the lowest concentrations of thyroxine; if this relationship also holds in vivo, it would tend to ensure that the foetus has an adequate supply of hormone if, for any reason, there is a deficiency. It should also be noted that if the foetus needs thyroid hormone, a substance in foetal blood with a high affinity for thyroxine would be equally important, whether the foetus uses its own hormone, or whether it relies on the mother for its supply. In either case, as long as the placenta were permeable to free thyroxine, the foetal blood would be unable to retain hormone if it did not contain a binding substance.

In man, it is not possible to study the partition of thyroxine between maternal and foetal serum in vitro by the method used for rabbits, because TBP in human foetuses cannot be distinguished from that in adults. However, the thyroxine-binding capacity of TBP in the serum from foetuses at the 15th week has been shown to be much lower than that of maternal serum (Osorio and Myant, unpublished). To this extent, the explanation we have put forward for the findings in rabbits could apply equally to human beings.

3. Passage of Thyroid Hormone from Foetus to Mother

So far, we have said nothing about the passage of thyroid hormone from the foetus to the mother. Nevertheless, the assumption that the hormone can cross the placenta in both directions is implicit in our attempt to explain some of the facts in terms of a competition for thyroxine between two binding proteins separated by a barrier permeable to free thyroxine. Unfortunately, there is very little reliable evidence on this point. From time to time it has been claimed that untreated myxoedema in women is temporarily relieved during pregnancy, and this has been put forward (Zondek, 1940) as showing that the hormone secreted by the foetus is available to the mother. Hall & Myant (1956) injected radioactive thyroxine into rabbit foetuses and found very little radioactivity in the maternal serum four hours later. However, under these conditions the concentration in the maternal serum would be expected to rise slowly, since the volume of maternal blood is so much larger than the volume of foetal blood.

4. Requirement of the Foetus for Thyroid Hormone

Although it is clear that the hormone secreted by the mother becomes increasingly available to the foetus at the end of pregnancy, it does not follow that this has any influence on foetal development. The earliest stages of growth and differentiation presumably take place in the absence of thyroid hormone, since maternal hormone appears to be unable to reach the foetus during this period, and the foetal thyroid has not begun to function. The fact that thyroidectomized animals are sometimes able to conceive and bear more or less normal young (Simpson, 1913; Hammett, 1922; Davenport & Swingle, 1927; Chu, 1944) also shows that development can take place without thyroid hormone until the time at which the foetus begins to make its own. There are several reports of untreated myxoedematous women who became pregnant and bore normal children, which give further support to this conclusion (Parkin & Greene, 1943; Hodges, Hamilton & Keettel, 1952; and see also the case of Lister & Ashe (1955), in which treatment was not begun until the fourth month).

With regard to the later stages of pregnancy, most of the available evidence suggests that normal foetal development

requires the presence of thyroid hormone within the foetus, though there is disagreement about this (Moore, 1950). The frequent occurrence of abortion and resorption of the foetus when the mother is thyroidectomized (Chu, 1944) or treated with thiouracil (Jones, Delfs & Foote, 1946; Krohn & White, 1950) may only mean that the mother requires thyroid hormone to maintain the proper environment for the foetus. It does not prove that the foetus itself requires a supply of hormone. However, the experiments of Peterson & Young (1952), which we have already mentioned, show that the foetal thyroid does not develop normally if the foetus is deprived of thyroid hormone. Weiss & Noback (1949) have also shown that if the mother is treated with thiouracil, ossification in the foetus is delayed—a defect which is almost certainly due to a deficiency of thyroid hormone within the foetus. Dorff (1934) has reported a case of congenital aplasia of the thyroid in a new-born child whose bones showed changes indicative of thyroid deficiency. This observation not only shows that the human foetus requires thyroid hormone. It also suggests that the mother cannot provide the foetus with an adequate supply.

5. Function of the Foetal Thyroid

Even if the foetus does require thyroid hormone for the final stage of its development, there remains the possibility that its own thyroid supplies it with all that it needs. Judged by the ability to synthesize hormone, the thyroid begins to function in foetal life. It is often assumed that because synthesis of hormone occurs, secretion must also occur. But this is not necessarily the case in foetal life, since the thyroid accumulates a store of colloid during the course of its development. Few attempts have been made to test this assumption. Jost, Morel & Marois (1949) showed that, if radioiodide is injected into rabbit foetuses in utero, the concentration of radioactivity in the foetal thyroid at 24 hours after the injection is much lower than at 1 hour. They took this as evidence for rapid turnover of thyroid hormone in the foetal thyroid. However, they did not show that the fall in radioactivity was due to loss of radioactive thyroxine from the thyroid. Using rabbits at the same stage of pregnancy as those used by Jost *et al.* (1949), we have found scarcely any fall in the concentration of radioactive thyroxine in the thyroids of foetuses taken on successive days from pregnant rabbits injected with radioiodide (Osorio and Myant, unpublished), even when the re-entry of radioiodide into the foetal thyroids is blocked. More convincing evidence for secretion of thyroxine by the foetal thyroid is the observation of Geloso (1958) that radioactive thyroxine appears in the foetal serum of rabbits injected with radioiodide, even if the mother's thyroid is removed before the injection. We have also found that in pregnant rabbits injected with radioiodide at the 28th day the specific activity of the radiothyroxine in the foetal serum reaches a higher value than that in the maternal serum (Osorio and Myant, unpublished).

The stage of pregnancy at which synthesis of hormone is first detectable in the foetal thyroid varies widely in different species. In rats, for instance, it is not until two or three days before the end of pregnancy that hormone is detectable in the foetal thyroid (Hall & Kaan, 1942), or that the thyroid can synthesize radioactive thyroxine (Waterman & Gorbman, 1953; Geloso, 1956), whereas in human beings synthesis may be assumed to begin during the fourth month (Chapman, Corner, Robinson & Evans, 1948; Hodges, Evans, Bradbury & Keettel, 1955), if the ability to fix radioiodide in a water-insoluble form is taken as proof of hormone synthesis. However, this discrepancy seems to be due more to differences in the degree of maturity of the young at birth than to differences in the maturity of the thyroid at a given stage of the development of the foetus as a whole. New-born rats are not nearly as mature as new-born children. There is certainly no reason to think that the foetal thyroid could supply the foetus with hormone at a particular stage of development in one species, but not in another. In the case of the skeleton, for instance, the thyroid begins to function both in rats and in man shortly after the appearance of the first centres of ossification (compare dates of ossification given by Strong (1925) and Mall (1906) with dates of functioning of the foetal thyroid given above).

As to the importance of the foetal thyroid as a source of hormone for the foetus, it is surprising that so few observations have been made on the effect of removing the thyroid from the foetus in utero. Jost (1957) says that removal of the thyroid of a rabbit foetus has no effect on the general growth of the foetus, or on the organization of its genital tract, though it does cause an increase in the total amount of foetal lipid (Jost & Picon, 1958). However, no one seems to have studied the effect of thyroidectomy in utero on the development of tissues known to be influenced by thyroid hormone in post-natal life, such as the nervous system and the skeleton. The observation of Dorff (1934) suggests that a detailed study of the effects of thyroidectomy in utero would be worth making in experimental animals.

6. Conclusion

In conclusion, it must be admitted that few of the questions we have raised can be answered with any certainty. Thyroid hormone is probably required by the foetus at some stage of its development. In some species, maternal hormone reaches the foetal circulation towards the end of pregnancy, and there are reasons for thinking that the partition of hormone between mother and foetus depends to some extent on the concentration of thyroxine-binding substances in the blood. However, it is not clear how far the maternal supply is essential to the foetus, since the foetal thyroid may also secrete hormone.

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THE HYPOTHALAMUS AND THE THYROID GLAND

K. BROWN-GRANT M.A. M.D.

Department of Physiology
University of Birmingham

1 Thyrotrophic activity of the autonomous pituitary
2 Effects of hypothalamic lesions and stimulation on thyroid function
3 Experimental analysis of hypothalamus–pituitary–thyroid interactions
  a Effect of exogenous thyroid hormone on thyroid function
  b Response to a deficit of thyroid hormone
  c Exposure to cold
  d Effect of "stress" on thyroid activity
4 Possible mechanisms for the regulation of thyrotrophin secretion
  a Regulation by systemic blood level of thyroid hormone
  b Regulation by the "effective" blood hormone level
  c Regulation by a neurohumoral transmitter
5 Influence of other areas of the central nervous system on thyroid gland activity
6 Conclusions
References

Stimuli which act through the central nervous system (CNS) to modify endocrine activity do so principally via changes in the rate of secretion of pituitary trophic hormones (Harris, 1948), and the hypothalamus and the hypothalamo-hypophysial portal vessels play an important role in this control (Harris, 1955a). In the present paper, the evidence for these views with respect to the thyroid gland will be discussed, and the extent to which the thyroid is normally under hypothalamic control, the mechanism of this control and the role of other parts of the CNS will be considered.

1. Thyrotrophic Activity of the Autonomous Pituitary

Separation of the pituitary from the CNS may be accomplished by section of the pituitary stalk, precautions being taken against the regeneration of the portal vessels, or by transplantation to a site in the body remote from the median eminence. After such operations the activity of the thyroid gland, as assessed by weight, histology and ^{131}I metabolism, is reduced, though not to the level seen after hypophysectomy. This has been observed after section of the pituitary stalk in the rabbit, rat and ferret and after transplantation of the gland in rat, rabbit, mouse, guinea-pig and hamster (references in Brown-Grant, Harris & Reichlin, 1957; Knigge & Bierman, 1958). Gonadal activity is reported as being severely depressed in such animals; adrenal activity, though reduced, is at a higher level than after hypophysectomy. A possible objection to experiments of this type is that the changes observed may be the result of non-specific damage due to ischaemia. The results of Harris & Jacobsohn (1952) are not easily explained on these grounds; they showed that pituitary tissue transplanted beneath the median eminence maintained normal thyroid weight and histology, whereas equally well-vascularized transplants beneath the temporal lobe of the brain failed to do so. More recently the specific effect of revascularization by the portal vessels has been conclusively demonstrated by Nikitovitch-Winer & Everett (1958). These workers first confirmed that grafts of pituitary tissue beneath the capsule of the kidney in the hypophysectomized rat showed only slight adrenocorticotrophic hormone (ACTH) and thyroid-stimulating hormone (TSH) activity, marked luteotrophic activity, and no follicle-stimulating hormone activity. Functional and histological studies were then made on animals in which the graft was left in the kidney, or retransplanted beneath the temporal lobe or beneath the median eminence. The "doubly insulted" gland, placed beneath the median eminence and revascularized by portal vessels, maintained normal thyroid weight and histology, and the uptake of ^{131}I was eight times greater than that of animals with grafts beneath the temporal lobe. Adrenal weight was also normal and oestrous cycles were resumed.

Thyroid activity is markedly reduced when the normal vascular relationship of the anterior pituitary to the hypothalamus is interrupted, though not to the level seen after hypophysectomy, indicating that the autonomous pituitary retains a limited capacity for TSH secretion.

2. Effects of Hypothalamic Lesions and Stimulation on Thyroid Function

Definite evidence of a reduction in thyroid activity as a result of electrolytic lesions of the hypothalamus has been obtained in the rabbit (Fortier & McDonald, personal communication, 1955), the dog (Ganong, Fredrickson & Hume, 1955), the rat (Greer, 1952; Bogdanove & Halmi, 1953; D'Angelo & Traum, 1956; Greer & Erwin, 1956; Reichlin, 1957; Florsheim, 1958; D'Angelo, 1958), the hamster (Knigge & Bierman, 1958), the guinea-pig (Bogdanove & D'Angelo, 1959), and the ferret (Donovan & van der Werff ten Bosch, 1959). There is a considerable measure of agreement that effective lesions are those bilaterally placed in the anterior basal hypothalamus (Harris, 1955b). Several of these workers have noted deficiencies of a single trophic hormone in their animals. Thyroidal hypofunction is not necessarily associated with gonadal atrophy or disturbance of adrenal or posterior pituitary activity. Such observations argue against the contention that the endocrine changes following hypothalamic lesions are non-specific in nature.

Early attempts to produce an increase in thyroid activity by stimulation of the CNS are reviewed by Harris & Woods (1958). In their own experiments on rabbits, a wire coil connected to unipolar or bipolar electrodes in the hypothalamus was implanted beneath the skin some weeks before the experiments were begun. Stimulation of the conscious, unrestrained animal was obtained by means of a large primary coil surrounding the animal's cage. They were able to produce an increase in the rate of release of thyroidal ^{131}I associated with a rise in the plasma level of labelled hormone, by stimulation of animals in which the electrode tips were in the anterior hypothalamus in the region of the supra-optico-hypophysial (SOH) tract, i.e., in the area where lesions are found to affect thyroid function. An interesting feature of their work is that animals in which the electrode tip was situated more posteriorly in the hypothalamus, that is, in the region where electrical stimulation has been shown to produce

ACTH release, gave negative results or even inhibition of ^{131}I release on stimulation. After adrenalectomy, more than one-third of the animals in which the electrode tip was in the median eminence showed a positive thyroid response to stimulation. Animals in which the electrode was posterior to the median eminence or in the pituitary gland itself did not show a positive thyroid response even after adrenalectomy. An overlap of the areas in which stimulation causes an increase in TSH and ACTH secretion is postulated, with the ACTH response in some way suppressing the thyroid response in animals with intact adrenal cortices. The situation is a complex one and no completely satisfactory explanation of this finding has yet been given. In more recent studies, using a technique similar to that of Soderberg (1958) in which the protein-bound radioiodine ($PB^{131}I$) in thyroid vein blood is measured in the anaesthetized animal at 10–20-minute intervals during an acute experiment, Campbell, George & Harris (1959) have confirmed their previous findings. An increase in thyroid activity has been observed 20–30 minutes after stimulation of the anterior hypothalamus in the region of the SOH tract. The time-course of the response is often indistinguishable from that observed after intravenous TSH injections, suggesting that the delay in activation of the pituitary must be measured in minutes, or perhaps even seconds, as has been shown to be the case for ACTH release (Gray & Munson, 1951) and has been surmised previously from the time-course of certain thyroid responses (Brown-Grant, von Euler, Harris & Reichlin, 1954; Reichlin & Reid, 1955).

3. Experimental Analysis of Hypothalamus–Pituitary–Thyroid Interactions

a. Effect of Exogenous Thyroid Hormone on Thyroid Function

The administration of thyroid hormone to the normal animal or human being results in a prompt, marked and reversible decrease in thyroid activity (Brown-Grant, 1957; Johnson, Solomon & Greer, 1959). Similar results are obtained in the rabbit after sectioning the pituitary stalk, but no changes are observed in the hypophysectomized rabbit nor is there any change in the sensitivity of the gland to exogenous TSH (Brown-Grant *et al.* 1957). Thyroxine administration further reduces the thyroid activity of rabbits (von Euler & Holmgren, 1956b) or mice (Scow & Greer, 1953) with grafts of pituitary tissue in the anterior chamber of the eye. A direct action of thyroxine on the adenohypophysis appears to be involved and it has been shown that a dose of thyroxine that has no effect when given systemically produces a decrease in thyroid activity when injected directly into the anterior pituitary of the rabbit (von Euler & Holmgren, 1956a). This experiment has also been performed on rats with similar results (Yamada & Greer, 1959), except that a delayed response (8–9 hours) to intra-hypothalamic injection of thyroxine was observed in contrast to the rabbit experiments where injections in the CNS itself were ineffective. Greer interprets these findings as indicating the presence of a thyroxine-sensitive receptor in the anterior hypothalamus of the rat. The long delay in the onset of inhibition is puzzling. When the same index of thyroid activity is used—the release of ^{131}I from the thyroid—the latency of the response to several far less direct stimuli has been found to be less than this (Brown-Grant, 1956a, 1956b).

A direct action of thyroxine upon anterior pituitary tissue, resulting in a decrease in TSH secretion, has been demonstrated. It is not necessary to invoke an action upon the hypothalamus in the intact animal to explain the results obtained, but such an action cannot be excluded.

b. Response to a Deficit of Thyroid Hormone

Interference with thyroid hormone synthesis by the administration of goitrogenic drugs of the thiouracil group leads to a lowering of the circulating thyroid hormone level. The subsequent structural and functional changes in the thyroid gland, which do not occur in the absence of the pituitary, may be taken as indicating an increased secretion of TSH. Lesions in the anterior hypothalamus of the rat and guinea-pig or transplantation of the pituitary in the mouse have been shown to reduce greatly, or abolish, the increase in weight that normally occurs during thiouracil administration. The increased ability of the gland to concentrate inorganic iodide (the thyroid : serum or T : S ratio) is only slightly or not at all affected (Greer, 1951, 1952; Bogdanove & Halmi, 1953; Scow & Greer, 1953; Bogdanove & D'Angelo, 1959). On the basis of this dichotomy between the growth and the T : S ratio response, Greer has postulated the existence of two thyrotrophins, one concerned with growth whose release is dependent upon normal vascular connexions with an intact hypothalamus, and a second metabolic TSH independent of CNS control. This hypothesis has been criticized on the grounds that the response of the T : S ratio, but not the morphological response to TSH, is potentiated by thiouracil (Halmi, Spirtos, Bogdanove & Lipner, 1953) and that the T : S ratio may be influenced by changes in intra-thyroidal iodine concentration independently of any action of TSH (Vanderlaan & Caplan, 1954). Greer (1957) did not accept these objections to his thesis, and he was supported in this by the finding of a similar dichotomy between the T:S ratio and the weight response in rats with hypothalamic lesions subjected to hemithyroidectomy (Reichlin, 1957), where the complicating factors mentioned above were not operative. However, D'Angelo (1958), using indices other than the T : S ratio, found impairment of both functional and morphological changes in rats in which lesions had been produced in the hypothalamus by electrolysis, while Bogdanove & Bogdanove (1957) have shown that the T : S ratio is far more sensitive to low levels of TSH stimulation than is the morphological response in rats. It seems clear that the different effect of the lesions on the two responses is a result of the far greater sensitivity of the T : S ratio to the reduced level of TSH secretion elicited in the animals which have undergone operation. Greer (1959) has recently published similar data on the sensitivity of the two responses in the mouse and now appears to agree that the results can be explained on the basis of a single TSH secreted in reduced amounts.

The response of the pituitary to a lowered level of circulating thyroid hormone is reduced but not abolished in animals where the normal vascular relationship to an intact hypothalamus is interrupted.

c. Exposure to Cold

A lowered environmental temperature is well known to increase thyroid activity; at least the acute response to this stimulus appears to involve the nervous system obligatorily. Uotila (1939) and Brolin (1946) showed that section of the pituitary stalk abolished the histological response of the rat thyroid to exposure to cold. Von Euler & Holmgren (1956b) showed that the usual increase in the rate of release of thyroidal

^{131}I did not occur when rabbits with anterior pituitary grafts in the eye were exposed to cold, while Knigge & Bierman (1958) obtained similar results in hamsters with pituitary grafts in the cheek pouch or with large bilateral lesions of the median eminence. The findings of Barrnett & Greep (1951), of a definite but reduced response of the thyroid after seven days' exposure to cold of rats in which the pituitary stalk had been cut, may indicate the possibility of a slower response mediated through changes in blood hormone content at the pituitary level. However, the possibility of regeneration of the portal vessels in their experiments cannot be excluded. The acute response of the thyroid to cold is dependent upon the presence of a pituitary in normal vascular relationship to an intact hypothalamus.

d. Effect of "Stress" on Thyroid Activity

The term "stress" is used here to describe any experimental procedure known to cause an increase in adrenocortical activity as the result of a reflex discharge of ACTH from the pituitary. Such procedures lead to a decrease in thyroid activity in the rabbit and the rat (Brown-Grant, Harris & Reichlin, 1954a; Brown-Grant, 1956a). A recent report that guinea-pigs and rhesus monkeys respond to stress with an increase in thyroid activity was based on the use of only one type of stimulus—bacterial exotoxins (Gerwing, Long & Pitt-Rivers, 1958). At least in the guinea-pig other stimuli (loud noises, adrenaline, typhoid vaccine) result in a decrease in thyroid activity (Brown-Grant & Pethes, 1960). The thyroid response to stress is thought to be due to a decrease in TSH secretion coincident with the increased secretion of ACTH and perhaps consequent upon it. It is not due to increased adrenal steroid secretion per se as it occurs in adrenalectomized animals (Brown-Grant, Harris & Reichlin, 1954a, 1954b; Brown-Grant, 1956a). The adrenal response to certain stress stimuli is abolished by transplantation of the pituitary, and Fortier (1951) has suggested that two types of stress may be distinguished: neurotropic stresses, the response to which is dependent on normal hypothalamic–pituitary connexions, and systemic stresses which act on the transplanted pituitary or in the presence of median eminence lesions.

In a series of experiments on rabbits whose pituitary stalks had been cut, it was found that the increase in adrenal activity and the decrease in thyroid activity which normally follow surgical trauma were not abolished by this procedure, whereas the divergent responses of these two glands to emotional stress (forced immobilization) were abolished. The adrenal response to cold, and the thyroid response to large doses of stilboestrol, were also abolished by section of the stalk, but not the adrenal response to adrenaline (Brown-Grant *et al.* 1957; Fortier, Harris & McDonald, 1957).

A normal vascular relationship between hypothalamus and pituitary is necessary for the thyroid and adrenal responses to certain stress stimuli.

4. Possible Mechanisms for the Regulation of Thyrotrophin Secretion

It has been established that the autonomous pituitary can maintain a reduced level of TSH secretion; that such a gland can respond in an appropriate manner to a raised blood level of thyroid hormone, to a reduced extent to a decrease in circulating hormone level and to certain stress stimuli. The pituitary with the normal vascular relationship to an intact hypothalamus maintains a higher basal level of thyroid activity and is capable of responding to additional stimuli—both stimulatory (cold) and inhibitory (emotional stress). Electrical stimulation of the hypothalamus can increase thyroid activity. These findings must be considered in any attempt to discuss the mechanism of the pituitary control of thyroid gland function.

a. Regulation by Systemic Blood Level of Thyroid Hormone

The ability to respond to changes in the level of circulating thyroid hormone appears to be an intrinsic property of anterior pituitary tissue, and many of the observed changes can be explained on the basis of a simple feed-back mechanism. However, certain stimuli (emotional stress, cortisone, stilboestrol) cause a decrease in the rate of release of ^{131}I from the thyroid gland of the rabbit that is associated with a fall in the level of circulating thyroid hormone rather than the rise that would be necessary if the change in activity were due to a simple feed-back mechanism. Further, the acute response to cold occurs before any change in blood hormone level would be expected, nor is it established that there is a reduction in the blood level of hormone during the period of sustained thyroid hyperactivity resulting from chronic exposure to cold (Brown-Grant, 1957). Clearly, a simple feed-back mechanism cannot account for the observed changes in thyroid activity.

b. Regulation by the "Effective" Blood Hormone Level

Analyses based on conventional measurements of the systemic blood level of thyroid hormone may, however, be misleading in certain circumstances. Recent studies (discussed by Tata, p. 142 of this number of the Bulletin) have shown that the blood, and possibly certain tissues, contain specific thyroxine-binding protein or proteins (TBP). At least one well-documented situation exists in man where changes in TBP may interfere with the normal feed-back mechanism. The rise in the blood concentration of protein-bound iodine (PBI) during pregnancy is well known and recent studies with ^{132}I have confirmed that the thyroid is hyperactive. There is no evidence to suggest an increased sensitivity of the gland to TSH (Halnan, 1958), and the pituitary reacts normally to an excess of exogenous thyroid hormone (Halnan, 1958; Werner, 1958). In the presence of a raised blood level of hormone and an increase in thyroid activity there are no signs of hyperthyroidism nor of an increase in basal metabolic rate beyond that which can be accounted for by the presence of an actively metabolizing mass of foetal tissue. The explanation may lie in the recent demonstration that either the level of TBP in the blood or the affinity of the protein for thyroxine is greatly increased during pregnancy (Dowling, Freinkel & Ingbar, 1956); as these authors suggest, such changes may alter the rate and extent to which thyroxine enters the maternal tissues. Robbins & Nelson (1958) present calculations suggesting that the level of free thyroxine in the plasma of pregnant women may actually be lower than normal because of the changes in TBP. The absence of symptoms and signs of hyperthyroidism may be due to failure of thyroid hormone to reach the maternal tissues in normal amounts; similarly, failure to reach the pituitary may explain the paradoxical situation of an excess of TSH leading to thyroid over-activity in the presence of a raised blood hormone level. It is significant that the hormone chosen to demonstrate the normal

reactivity of the pituitary during pregnancy was 3 : 5 : 3′-triiodothyronine, which is known to be much less firmly bound to TBP than thyroxine (Tata, p. 142 of this number of the Bulletin). Much remains to be discovered about possible variations in TBP level. However, such changes merit consideration when changes in thyroid activity occur in association with changes in the concentration of PBI in the blood, which appear to contradict the general concept of a feed-back regulation of the pituitary–thyroid axis.

The second mechanism by which the "effective" blood hormone level to which the pituitary is exposed might differ from the systemic blood level is represented by the hypothalamo-hypophysial portal vessel system. In many species this represents the major blood supply to the adenohypophysis (Harris, 1955a). There is evidence in some species that thyroxine is concentrated in the median eminence and posterior pituitary and it is conceivable that this may represent a removal of thyroxine from the blood during its passage through the capillaries of the primary plexus of the portal system. The hypothalamus could act as a filter, with the result that the anterior pituitary receives blood of reduced hormone content; variations in extraction rate as a result of changes in the hypothalamus would provide a stimulus in the form of local variations in blood hormone content, to which the pituitary is known to be capable of responding by an increase or decrease in TSH secretion. These speculations have been discussed in more detail elsewhere (Brown-Grant, 1957) but they have not yet received any direct experimental support.

c. Regulation by a Neurohumoral Transmitter

An alternative role for the portal vessels is the transmission of some neurohumour elaborated in appropriate amounts by the hypothalamus that would act upon the pituitary to stimulate TSH secretion (Harris, 1955b). The nature of the neurohumoral transmitter substance or substances for TSH or for any of the trophic hormones is still unknown. The current view is that vasopressin may be concerned in the release of ACTH (but see reviews by Donovan & Harris, 1957; Fortier & de Groot, 1958), while oxytocin has been implicated in the release of prolactin (Cowie & Folley, 1957; McCann, Mack & Gale, 1959). A vasopressin-like substance has been suggested as a possible agent for TSH release (Dubreuil & Martini, 1956; Harris & Woods, 1958). There is little direct evidence at present to support this view. Against it are the possible specific role of vasopressin in ACTH release and the evidence, presented in section 3d, of a reciprocal relationship between ACTH and TSH secretion. In my unpublished experiments, high doses of commercial vasopressin (5 units per day), which are known to cause ACTH release, have been found to inhibit the release of ^{131}I-labelled hormone from the thyroid gland of rabbits and guinea-pigs.

5. Influence of other Areas of the Central Nervous System on Thyroid Gland Activity

Some information on this point has been obtained from experiments in which parts of the CNS have been removed. Greer & Shull (1957) have shown that the neocortex is not essential for the response to thiouracil in the rat. Bard & Woods (personal communication, 1958) have studied cats after the removal of cerebrum, thalamus and much of the hypothalamus and observed normal thyroid activity as judged by ^{131}I studies and the response to thyroxine some weeks after operation; the existence of permanent polyuria in these animals is additional evidence for near-normal levels of thyroid activity. Cats with a small island of hypothalamic tissue separated from the rest of the CNS but attached to the pituitary also show normal thyroid activity. In clinical studies no striking changes in thyroid activity have been reported after leucotomy or removal of portions of the cerebral cortex (Reiss, 1954). A greater variability in the results of tests of thyroid function with ^{131}I in psychiatric patients has been reported, but no clear correlation of hypofunction or hyperfunction with the clinical diagnosis was established. There was some suggestion of a return towards more normal values in those patients who showed a favourable clinical response to treatment (Reiss, Hemphill, Maggs, Haigh & Reiss, 1953).

The possibility that emotional disturbances, acute or chronic, acting through the hypothalamus to produce a hypersecretion of TSH, may play a part in the aetiology of Graves's disease is still under discussion. Animal experiments in which complex emotional stimuli were employed (e.g., repeated changes of caging conditions) (Carrière & Isler, 1959) have consistently shown a decrease in thyroid activity. The report of Kracht & Kracht (1952) that a state of "fright thyrotoxicosis" occurs in wild rabbits captured by ferreting and kept under laboratory conditions could not be confirmed (Brown-Grant *et al.* 1954a). Further, the basis of this hypothesis, that Graves's disease is the result of pituitary over-activity, is not well established. The response of the thyroid in Graves's disease to exogenous TSH, and the failure of thyroxine to depress its activity, suggest that endogenous TSH activity is already suppressed in this condition (Werner, Spooner & Hamilton, 1955) and that Graves's disease may be an autonomous thyroid disorder. Recent reports of hyperthyroidism in patients with non-functioning chromophobe adenomata of the pituitary or post-partum necrosis of the pituitary, and after hypophysectomy (reviewed by Gurling, Baron & Smith, 1959), support this view.

6. Conclusions

As this review has indicated, our knowledge of the role of the nervous system in the control of thyroid gland activity has been greatly extended in recent years. The precise means by which this control is exerted has not yet been established, though certain possibilities amenable to experimental investigation have been suggested. The hypothalamus–pituitary–thyroid system is of prime importance in the study of the physiology of the thyroid; however, other mechanisms which are at present dismissed as having no significant role in the control of the gland in normal animals may be of greater importance in pathological conditions of the gland and for this reason deserve to receive more critical attention from the experimentalist.

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Notes on Contributors

DR D. D. ADAMS is a research officer in the New Zealand Medical Research Council's Endocrinology Research Department. In 1954 he was a lecturer in Professor J. B. Collip's Department of Medical Research at the University of Western Ontario in Canada. He has worked mostly on problems relating to the thyroid gland, and his major interest at present is the aetiology of Graves's disease. Dr Adams also does some clinical work in endocrinology. In 1958 he was awarded the Van Meter Prize Award of the American Goiter Association. Among his publications are: "The presence of an abnormal thyroid-stimulating hormone in the serum of some thyrotoxic patients" (*J. clin. Endocrin. Metab.* 1958, **18**, 699); with H. D. Purves, "Thyrotrophin assay by plasma I^{131} measurements" (*Canad. J. Biochem. Physiol.* 1957, **35**, 993); and, with H. D. Purves, "The change in thyroidal I^{131} content between 8 and 48 hours as an index of thyroid activity" (*J. clin. Endocrin. Metab.* 1957, **17**, 126).

DR G. S. BOYD was appointed Assistant in the Department of Biochemistry, University of Edinburgh, in 1948; he studied problems associated with biological methylation for two years and obtained a Ph.D. in 1951. He then started work on cholesterol metabolism in man and in experimental animals, with special reference to the possible endocrine influences in cholesterol metabolism. The investigation in human beings has mainly centred on the study of the correlation of the alleged defect in lipid metabolism associated with atherosclerosis. Subjects of Dr Boyd's recent papers include the role of coenzyme A in cholesterol biosynthesis, serum cholesterol changes during the menstrual cycle, and the influence of hormones on the serum cholesterol level in man and animals. He has previously contributed to the Bulletin a paper entitled "Hormonal control of the circulating lipids" (*Brit. med. Bull.* 1958, **14**, 239, with M. F. Oliver).

DR K. BROWN-GRANT was educated at Cambridge where he was a Scholar of Gonville and Caius College, and qualified from St Mary's Hospital, London, in 1951. From 1952 to 1955 he held a Medical Research Council Studentship and worked under Professor G. W. Harris in the Department of Physiology, Cambridge, and then at the Institute of Psychiatry, London. During his military service in the Royal Army Medical Corps he worked as a pathologist and later worked at the Chemical Defence Experimental Establishment of the Ministry of Supply at Porton, Wiltshire. In 1957 he was appointed Lecturer in Physiology in the University of Birmingham and spent a year studying steroid biochemistry as a United States Public Health Service Fellow at the Worcester Foundation, Massachusetts, before taking up his present position. His research has been concerned with the physiology of the thyroid gland, particularly its relationship to the other endocrine glands, and the distribution and metabolism of iodine in the body. Dr Brown-Grant is also interested in the problems of the response of the whole animal to trauma and in the experimental approach to the study of psychosomatic disorders.

DR F. W. CLEMENTS has been Assistant Director, Institute of Child Health, University of Sydney, since 1951. From 1938 to 1949 he was Director, Australian Institute of Anatomy, Canberra, and Chief of the Nutrition Unit, Commonwealth Health Department. His interest in endemic goitre started with an investigation of its extent in Canberra, following which the Government introduced a programme of iodine prophylaxis which has effectively controlled the condition there. From 1949 to 1951 he was Chief, Nutrition Section, World Health Organization, Geneva, at the time the Organization launched its world-wide programme of goitre prevention. In 1952 he was Chairman of the World Health Organization Study Group on Endemic Goitre which met in London. For the last few years Dr Clements has been following up the unusual set of conditions associated with the occurrence of endemic goitre in parts of Australia, where, apparently, iodine prophylaxis is not effective. Among his publications are: "Goitre studies" (*Med. J. Aust.* 1948, **1**, 637, 665, 753); "The relationship of thyrotoxicosis and carcinoma of the thyroid to endemic goitre" (*Med. J. Aust.* 1954, **2**, 894); with J. W. Wishart, "A thyroid-blocking agent in the etiology of endemic goiter" (*Metabolism*, 1956, **5**, 623); "Endemic goitre: scope of the health problem and related conditions" (*Bull. World Hlth Org.* 1958, **18**, 175).

DR DEBORAH DONIACH received her medical training at the Royal Free Hospital, University of London, and qualified in 1944. She became Junior Lecturer in Clinical Biochemistry at the Courtauld Institute, Middlesex Hospital, in 1947. Here she received training in endocrinology and obtained the M.D. degree in chemical pathology in 1950. A research fellowship at the Royal Free Hospital between 1951 and 1954 allowed her to concentrate on clinical aspects of thyroid diseases as well as carry out isotope and metabolic studies. She made the clinical trials establishing carbimazole for the treatment of thyrotoxicosis and also became interested in Hashimoto's disease. Returning to the Middlesex Hospital to work as thyroid research fellow and clinical assistant to the thyroid clinic under Mr R. Vaughan Hudson, she undertook investigations which led to her collaboration with Dr Roitt and their joint work on thyroid auto-immunity. With Dr Roitt she was awarded the Van Meter Prize Award for 1957. Her published works include: "Treatment of thyrotoxicosis with 'Neo Mercazole' (2-carbethoxythio-l-methylglyoxaline): report of 120 cases" (*Lancet*, 1953, **1**, 873); with I. M. Roitt, "Human auto-immune thyroiditis: serological studies" (*Lancet*, 1958, **2**, 1027); with I. M. Roitt & R. V. Hudson, "Human auto-immune thyroiditis: clinical studies" (*Brit. med. J.* 1960, **1**, 365).

DR I. DONIACH, Reader in Morbid Anatomy, Postgraduate Medical School of London, was formerly cancer research assistant to Dr J. C. Mottram at Mount Vernon Hospital, London. His work is divided between morbid anatomy and experimental pathology. The experiments have been concerned with effects of radiation on the rat's thyroid gland, and various aspects of growth and regeneration of the thyroid. Publications include: with Dr J. H. Logothetopoulos, "Compensatory hypertrophy of the rat thyroid after partial thyroidectomy" (*Brit. J. exp. Path.* 1955, 36, 617); with Dr J. H. Logothetopoulos, "Effects of radioactive iodine on the rat thyroid's function, regeneration and response to goitrogens" (*Brit. J. Cancer*, 1955, **9**, 117); "Experimental induction of tumours of the thyroid by radiation" (*Brit. med. Bull.* 1958, **14**, 181).

DR J. T. EAYRS is the Henry Head Research Fellow of the Royal Society and Reader in Comparative Neurology in the University of Birmingham. His main interest has been in the interrelationship between the nervous and endocrine systems and in this field he has studied the impact of thyroid secretion on the development of the central nervous system and the neural factors involved in the regulation of lactation. He recently spent a year at the California Institute of Technology, engaged in psycho-biological research, and at the present time is continuing investigations into problems involving transmodal sensory integration and the functional plasticity of nervous tissues. Dr Eayrs' publications include: "The cerebral cortex of normal and hypothyroid rats" (*Acta anat.* 1955, **25**, 160); with R. M. Baddeley, "Neural pathways in lactation" (*J. Anat., Lond.* 1956, **90**, 161); and "The status of the thyroid gland in relation to the development of the nervous system" (*Anim. Behav.* 1959, **7**, 1).

DR W. HOWEL EVANS is the Senior Medical Registrar at the Liverpool Royal Infirmary. He graduated from Liverpool University in 1948. Since 1951 he has held several appointments as Registrar in Liverpool hospitals and is particularly interested in thyroid disease. He is a member of a team, headed by Dr C. A. Clarke, investigating the relationship of peptic ulcer to the ABO blood groups. In 1956–57 he investigated a Liverpool family in whom carcinoma of the oesophagus was found to occur frequently in those members who had inherited tylosis, a hereditary syndrome not previously described. At present Dr Howel Evans is working with Dr F. D. Kitchin on a family survey of thyroid disease, with reference to the phenylthiocarbamide status. His published work, with other authors, includes: "The relationship of the ABO blood groups to duodenal and gastric ulceration" (*Brit. med. J.* 1955, **2**, 643); "ABO blood groups and secretor character in duodenal ulcer: population and sibship studies" (*Brit. med. J.* 1956, **2**, 725); "Carcinoma of the oesophagus with keratosis palmaris et plantaris (tylosis): a study of two families" (*Quart. J. Med.* 1958, **27**, 413); "P.T.C. taste response and thyroid disease" (*Brit. med. J.* 1959, **1**, 1069).

DR A. W. G. GOOLDEN since 1952 has been at the Hammersmith Hospital, London, where he is now a consultant in radiotherapy and an honorary lecturer in radiotherapy to the Postgraduate Medical School of London. He has been attached to the Medical Research Council's Radiotherapeutic Research Unit since 1955. He is interested in radiation cancer and various aspects of thyroid metabolism. His publications include: "Radiation cancer: a review with special reference to radiation tumours in the pharynx, larynx, and thyroid" (*Brit. J. Radiol.* 1957, 30, 626); "The effect of thyrotropic hormone on the accumulation of radioactive iodine in thyrotoxicosis" (*J. clin. Endocrin. Metab.* 1959, **19**, 1252); and "A comparison of radioiodine tests in the diagnosis of hyperthyroidism" (*Brit. J. Radiol.* 1958, **31**, 433).

SIR CHARLES HARINGTON has been Director of the National Institute for Medical Research, London, since 1942. Before this, he was Professor of Chemical Pathology in the University of London from 1931 to 1942, and Director of the Graham Medical Research Laboratories, University College Hospital Medical School, London, from 1937 to 1942. He was elected a Fellow of the Royal Society in 1931, and was Croonian Lecturer of the Royal Society in 1944, when the title of his lecture was "Thyroxine: its biosynthesis and its immuno-chemistry". Sir Charles was a member of the Medical Research Council from 1938 to 1942, and editor of the *Biochemical Journal* from 1930 to 1942. He has published numerous papers on chemical and biochemical subjects, mainly in the *Biochemical Journal*.

NOTES ON CONTRIBUTORS

DR F. D. KITCHIN is Senior Registrar in Medicine at Broadgreen Hospital, Liverpool. He qualified in 1947 and after military service worked with Professor H. L. Sheehan in the Department of Pathology, Liverpool University. From 1956 until 1959 he was a medical registrar at the David Lewis Northern Hospital, Liverpool, where, in collaboration with Dr Howel Evans and under the direction of Dr C. A. Clarke, he studied the genetic aspects of thyroid disease. This work has been concerned with the taste response to phenylthiocarbamide (PTC) in various forms of thyroid disease and is the basis of his M.D. thesis. Currently Dr Kitchin is conducting a family study on this subject designed to elucidate the previous findings, and is investigating the taste reaction to PTC in various laboratory animals. His publications include: "P.T.C. taste response and thyroid disease" (*Brit. med. J.* 1959, **1**, 1069), written in association with Dr Howel Evans, Dr C. A. Clarke, Dr R. B. McConnell and Dr P. M. Sheppard.

DR E. M. MCGIRR graduated in science and medicine at Glasgow, and is now Senior Lecturer in Medicine at the University Department of Medicine, Royal Infirmary, Glasgow. Since 1949 he has been interested in the application of radioactive isotopes to clinical medicine, particularly in peripheral vascular, thyroid and blood disorders. His present work is chiefly concerned with elucidating the various biochemical anomalies that occur in sporadic goitrous cretinism and sporadic familial goitre in young patients, and with their hereditary aspects. Dr McGirr has previously contributed to the Bulletin (*Brit. med. Bull.* 1952, **8**, 192) an article on tissue clearance of inorganic ions. His publications also include: "Hypothyroidism as an inborn error of metabolism" (*J. clin. Endocrin. Metab.* 1954, **14**, 869, with Dr James H. Hutchison); and "Sporadic or non-endemic familial cretinism with goiter" (*Amer. J. Med.* 1957, **22**, 712, with Dr J. B. Stanbury).

DR N. B. MYANT, a member of the Scientific Staff of the Medical Research Council, has been working at the Experimental Radiopathology Research Unit, at Hammersmith Hospital, London, since 1953. His investigations have been chiefly on problems connected with iodine metabolism. Dr Myant contributed a paper, "Radioiodine tests of thyroid function in man", to an earlier number of the Bulletin (*Brit. med. Bull.* 1952, **8**, 141).

DR M. F. OLIVER is Senior Research Fellow (Scottish Hospital Endowments Research Trust) and is a physician in the Department of Cardiology, Royal Infirmary, Edinburgh. Formerly he was Research Fellow (Advisory Committee for Medical Research) and Kirk Duncanson Research Fellow (Royal College of Physicians of Edinburgh), both in the same Department. For the past seven years he has been concerned in clinical and experimental investigations into the aetiology of coronary disease and its relationship to disturbances of lipid metabolism and of endocrine function. Among his publications are the following, all in collaboration with G. S. Boyd: "The plasma lipids in coronary artery disease" (*Brit. Heart J.* 1953, **15**, 387); "The influence of the sex hormones on the circulating lipids and lipoproteins in coronary sclerosis" (*Circulation*, 1956, **13**, 82); "Endocrine aspects of coronary sclerosis" (*Lancet*, 1956, **2**, 1273); and "Hormonal aspects of coronary artery disease" (*Vitam. and Horm.* 1958, **16**, 147). He has previously contributed to the Bulletin a paper entitled "Hormonal control of the circulating lipids" (*Brit. med. Bull.* 1958, **14**, 239, with G. S. Boyd). Dr Oliver is Honorary Secretary of the Atherosclerosis Discussion Group, and a member of the British Cardiac Society, of the Medical Research Society, and of other medical societies.

DR C. OSORIO graduated in medicine at the University of Santiago, Spain, where he was a lecturer in the Department of Physiology from 1955 to 1958. Since that time he has worked in the Experimental Radiopathology Research Unit of the Medical Research Council at Hammersmith Hospital, London. He is interested in the thyroid hormone.

DR ROSALIND PITT-RIVERS is a member of the Scientific Staff of the Medical Research Council, at the National Institute for Medical Research, London. Her main work has been on the biochemistry of the thyroid hormone, and she is continuing these investigations. She was awarded the William Julius Mickle Fellowship of the University of London for 1951–52, and elected Fellow of the Royal Society in 1954. Dr Pitt-Rivers worked with Dr J. B. Stanbury at the Massachusetts General Hospital, Boston, 1953–54, and with Dr J. E. Rall, National Institute for Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland, 1959–60. Her principal work is on the discovery of a new thyroid hormone, tri-iodothyronine, with Dr Jack Gross. In collaboration with Dr Gross, Dr Pitt-Rivers contributed a paper entitled "Experimental study of thyroid metabolism with radioactive iodine" to a previous number of the Bulletin (*Brit. med. Bull.* 1952, **8**, 136).

DR H. D. PURVES is a principal research officer of the New Zealand Medical Research Council and is Director of the Council's Endocrinology Research Department. His early work was on endemic goitre. Later he studied the mode of action of goitrogens, eventually showing them to be antithyroid drugs. His present work includes problems related to the thyroid and pituitary glands and the hypothalamus. In 1949 Dr Purves held a Rockefeller Fellowship, visiting the United States of America, and in 1956 he attended the Ciba Foundation Colloquium on Endocrinology in London. Among his publications are: "Studies on experimental goitre: IV. The effect of di-iodotyrosine and thyroxine on the goitrogenic action of *Brassica* seeds" (*Brit. J. exp. Path.* 1943, **24**, 171); with W. E. Griesbach, "The site of thyrotrophin and gonadotrophin production in the rat pituitary studied by the McManus-Hotchkiss staining for glycoprotein" (*Endocrinology*, 1951, **49**, 244); and, with D. D. Adams, "The role of thyrotrophin in hyperthyroidism and exophthalmos" (*Metabolism*, 1957, **6**, 26).

DR I. M. ROITT has been a research fellow at the Courtauld Institute of Biochemistry, Middlesex Hospital, London, since 1953. After early work on the mode of action of growth inhibitors, he became interested in problems of auto-immunity and has studied the nature and underlying mechanisms of auto-immune thyroiditis. Among his publications in this field are: with D. Doniach, "Auto-immunity in Hashimoto's disease and its implications" (*J. clin. Endocrin. Metab.* 1957, **17**, 1293: Van Meter Prize Award essay of the American Goiter Association); with P. N. Campbell & D. Doniach, "The nature of the thyroid auto-antibodies present in patients with Hashimoto's thyroiditis (lymphadenoid goitre)" (*Biochem. J.* 1958, **69**, 248); and, with R. J. V. Pulvertaft, D. Doniach & R. V. Hudson, "Cytotoxic effects of Hashimoto serum on human thyroid cells in tissue culture" (*Lancet*, 1959, **2**, 214).

DR J. R. TATA has been a Beit Memorial Fellow since 1956 at the National Institute for Medical Research, London. He was Visiting Research Fellow at the Sloan-Kettering Institute, New York, from 1954 to 1956. After working on the biochemistry of malarial parasites at the Indian Institute of Science, Bangalore, his main activities in biochemistry of thyroid hormones began in Paris in 1951. He is interested at present in thyroxine–protein interactions and the enzymic dehalogenation of thyroid hormones. Dr Tata is a co-author, with Dr R. Pitt-Rivers, of two monographs: *The thyroid hormones* (1959) and *The chemistry of thyroid diseases* (1960). He has recently described a transient physico-chemical instability of thyroxine (*Biochem. J.* 1959, **72**, 214, 222). He was awarded the Van Meter Prize Award in 1956 for work on iodoproteins in thyroid cancer.

MR SELWYN TAYLOR is Consultant Surgeon at King's College Hospital, London, and has been Lecturer in Surgery on the staff of the Postgraduate Medical School of London since 1947. He studied at Oxford and King's College Hospital Medical School, London, and from 1940 to 1946 served in the Royal Naval Volunteer Reserve, finally holding the rank of surgeon lieutenant-commander. In 1947 he was George Herbert Hunt Travelling Scholar of Oxford University, working with Professor C. Crafoord in Stockholm, and in 1948 was awarded a Rockefeller Travelling Fellowship and spent a year at the Massachusetts General Hospital, Boston. His particular interest has been in simple goitre, on which subject he has published a number of papers. He is Senior Honorary Editor of the *Proceedings of the Royal Society of Medicine* and edited the 1959 edition of *Recent advances in surgery*. He is Honorary Secretary of the Surgical Research Society and also of the Fourth International Goitre Conference which is being held in London in July 1960. His publications include: "The evolution of nodular goiter" (*J. clin. Endocrin. Metab.* 1953, **13**, 1232); "Calcium as a goitrogen" (*J. clin. Endocrin. Metab.* 1954, **14**, 1412); "The thyroid nodule" (*Lancet*, 1958, **1**, 751).

DR W. R. TROTTER has been attached to the Medical Unit, University College Hospital Medical School, London, either as assistant or as research worker, since 1937. In 1938 (with the late K. C. Eden) he started a clinic for patients with thyroid disease, and this work still continues (with M. E. Morgans). He has undertaken research on many aspects of thyroid disease, and is at present particularly interested in the immunological and genetic approaches to thyroid problems. He made a study (with K. C. Eden) of localized pretibial myxoedema in association with thyrotoxicosis (*Quart. J. Med.* 1942, **11**, 229); and (with G. Belyavin) of the nature of the thyroid antigens reacting with serum from patients with Hashimoto's disease (*Lancet*, 1959, **1**, 648). He has also contributed a chapter on diseases of the thyroid to the book by R. Pitt-Rivers & J. R. Tata entitled *The thyroid hormones* (1959).

Book Reviews

The Thyroid Hormones

Rosalind Pitt-Rivers & Jamshed R. Tata, with a chapter on diseases of the thyroid by *W. R. Trotter*. London: Pergamon Press, 1959. (International Series of Monographs on Pure and Applied Biology.) xiii + 247 pages; 22 figures. 23 × 16 cm. £2 10s.

The development of our knowledge of the thyroid gland forms a fascinating study and, although there are many earlier references, it dates principally from the beginning of the nineteenth century. At that time the clinical features of thyrotoxicosis were described, the element iodine was discovered, and the first investigations were made of its importance in the pathogenesis and treatment of goitre. By the end of the century physiological studies had clearly demonstrated the nature of myxoedema, and replacement therapy was introduced. It was again the turn of the chemist and successively there followed the demonstration of the high concentration of iodine in the thyroid gland, the isolation of thyroxine, and finally the elucidation of the structure of this hormone. Subsequently the introduction of chromatographic analysis and the availability of radioactive isotopes of iodine laid the path for further advances. In this way tri-iodothyronine has been demonstrated in the thyroid gland and in the plasma, and the formation, distribution and metabolism of thyroid hormones have been further elucidated. All these physiological and biochemical studies have been described in detail and critically reviewed in this excellent book. Pharmacological aspects have also been considered and there is a full account of the nature and action of substances which inhibit thyroid function.

The authors have provided an extensive review of the published work on their subject, but this book is far from a mere compilation of experimental observations and deductions. The judicious analysis of recent work and the emphasis on the important questions that are at present obscure make stimulating reading. Dr Pitt-Rivers herself played a large part in the isolation of tri-iodothyronine, and it has been suggested that thyroxine had to be de-iodinated to this compound before the typical peripheral effects became apparent. There is, however, a discriminating account of the reasons why this view is untenable and it is suggested that a search for rapidly acting derivatives of thyroxine may have led us in the wrong direction in the elucidation of thyroid hormone action. We are still ignorant of the mechanism by which iodine is concentrated in the thyroid and of the mechanism of action of the hormones, and recent investigations of these difficult topics are fully discussed. Here again the comments made by the authors are pertinent and thought provoking.

The book concludes with a chapter on thyroid diseases written by W. R. Trotter, and this is in keeping with the main theme. Attention is concentrated on the aetiology of various thyroid disorders. In spite of the great advances in the biochemical and physiological aspects, the causation of thyrotoxicosis and of sporadic goitre is still unknown; but there is an impressive review of the relevant evidence.

The authors are to be congratulated on producing a book which is certainly the best account of the subject available at the present time. It is clearly written, attractive in style and only occasionally marred by oversights in proof-reading. No serious investigator in the thyroid field can afford to be without it.

G. M. Wilson

Principles of General Physiology. Volume 1: The Physico-Chemical Background

L. E. Bayliss. 5th ed. London: Longmans, Green & Co. Ltd., 1959. xiii + 520 pages; figures. 24 × 16 cm. £2 15s.

Although this is nominally the fifth edition of Sir William Bayliss's *Principles of general physiology*, the text has been almost completely rewritten by Sir William's son, Dr L. E. Bayliss. The first edition appeared in 1915 and the fourth in 1924. In those editions there were several chapters and parts of chapters which dealt with the basic physical chemistry of biological systems. Since they appeared, however, there have been great changes in physico-chemical ideas, perhaps most significantly in the theory of electrolytes and of dilute solutions. These new ideas have had a profound bearing on physiology, particularly as the processes of biology mostly take place in dilute electrolyte solutions. With these changes in mind, Dr Bayliss thought it best to bring together all the physico-chemical background to biology into a separate first volume, leaving himself a second volume in which to deal more particularly with general physiology itself. This division of the book into two moderately sized parts certainly makes for greater ease in its use, and will doubtless appear less daunting than a single enormous tome.

In writing this book Dr Bayliss has assumed the reader to have an elementary knowledge of physics and chemistry, and to be familiar with elementary mathematics, including a nodding acquaintance with the infinitesimal calculus. The subjects covered include energy and thermodynamics; the kinetics of chemical reactions and their energy changes; the theory of solutions, osmotic effects, viscosity; and weak and strong electrolytes, acids and ampholytes. The section on surface phenomena treats surface energy, surface tension, monomolecular layers, adsorption and surface electrical phenomena. There are also sections on the colloidal state, catalysis and enzymes, oxidation-reduction systems, the actions of light and other radiations, and a final one on the passage of substances through membranes.

Since so wide a field has been covered, it is inevitable that there has been a great deal of compression. Perhaps one of the weaknesses of this volume is the enormous range of subjects dealt with: it might have been better to have left out some topics and to have expanded others. One also gains the impression that, in some sections at least, many concepts are included which have outlived their usefulness. Thus the section on enzyme-catalysed reactions has rather an old-fashioned character, a considerable amount of space being occupied by descriptions of such outmoded ideas as the Schütz rule and chain reactions. Most of the examples quoted in this section are from very old work—not that this necessarily means that they are invalid, but many of the recent significant changes which have occurred in our ideas on enzymes are hardly dealt with.

Despite these criticisms this is an excellent and interesting book, and will prove to be a mine of information and instruction for many biologists. It is not a book for light reading, but it will well repay study and it is written in a manner which enables one to grasp the essential point of the argument easily.

Finally it should be mentioned that at the end of each chapter there are numerous references to reviews and books where further information and inspiration may be found. After reading this volume, one is left impatiently awaiting the publication of the second half.

S. P. Datta

Isotopic Tracers: A Theoretical and Practical Manual for Biological Students and Research Workers

G. E. Francis, W. Mulligan & A. Wormall. 2nd ed. London: Athlone Press, 1959. xxi + 524 pages; 74 figures. 22 × 14 cm. £2 12s. 6d.

Training in isotopic tracer techniques now forms an essential part in the apprenticeship of every biological research worker, and the appearance of a revised and enlarged second edition of this deservedly popular text-book will be especially welcome to all concerned in the application of tracer methods to biochemistry. This volume is essentially a laboratory manual, and contains a wealth of practical information concerning methods of preparation, handling and assay of radioactive materials.

The first part of the book is concerned with theoretical considerations. After a brief introduction, five chapters deal clearly and concisely with the fundamental principles of radioactivity physics, the preparation of radioisotopes and methods of synthesis of labelled compounds. The next two chapters are concerned with general methods for the assay of stable and radioactive isotopes respectively. They are followed by two chapters dealing with the

principles governing the choice of radioactive assay technique in biological investigations and the interpretation of such assay measurements. The relative merits of different assay methods are clearly stated. Subsequent chapters are concerned with autoradiographic techniques, radiation hazards, and examples of the use of isotopic tracer techniques in biological research. Newly added sections include discussions of activation analysis and exchange labelling techniques with tritium.

The second part of the book gives details of 13 exercises comprising a practical course in radioactive isotope techniques. These exercises include the measurement of thyroid activity in terms of the protein-bound radioiodine in the blood after injection of ^{131}I, the localization of injected ^{131}I in the thyroid and the paper chromatography of ^{131}I-containing urines.

Throughout the book, the treatment of the subject is essentially practical. Much valuable and well-balanced advice is given concerning the relative merits of different assay systems. References are limited for the most part to text-books and review articles. It should be emphasized that this volume is written primarily for the laboratory worker and that the chapters dealing with uses of tracer techniques are concerned almost entirely with applications in the biochemical field. Medical diagnostic and therapeutic uses of radioisotopes are only briefly discussed, and physiological and physico-chemical uses receive only cursory mention.

A few minor criticisms may be recorded. The chapter dealing with radiation hazards comprises 30 pages concerned with theoretical aspects of radiation damage and only nine concerned with practical precautions in the use of radioactive materials. In a practical laboratory manual, more detailed information on the design and management of laboratories dealing with radioactive materials would be desirable. Certain of the recommendations which are quoted relating to maximum permissible radiation dose have now been rendered obsolete by the 1958 Recommendations of the International Commission on Radiological Protection. The examples of biological uses of isotopic tracers to study the kinetics of biological processes could be improved by the more liberal use of diagrams. However, exception cannot be taken to a few such deficiencies in a text-book dealing with a rapidly developing field, and this book can be warmly recommended to research workers in the biological sciences as an authoritative introduction to isotopic techniques.

E. H. Belcher

An Introduction to Medical Genetics

J. A. Fraser Roberts. 2nd ed. London: Oxford University Press, 1959. x + 263 pages; 107 figures. 25 × 17 cm. £1 15s.

The task of reviewing this book is difficult because there is no other with which it can be compared. There are excellent books on blood groups, coagulation defects, metabolic disorders, mental deficiency, and so on; there are books introducing the statistician to human data, the medical student to the fruit-fly, and the drosophilist to man. But since the first edition of this book 20 years ago there has been no lucid, sound and detailed book introducing man to man in the field of genetics.

Dr Fraser Roberts covers the main field of human genetics against a background of reliable pedigrees profusely illustrated by photographs and *x* rays. Most of the conditions are necessarily rare, but provide a more satisfactory introduction to medical practitioners, for whom this book is written, than do the conditions in various animals, insects or plants which form the usual background of the non-medical geneticist.

Various deficiencies may be noted by the specialist within the various branches, but even these seem wholly commendable in a book of 250 pages. Mathematical analysis and symbolism are almost absent: there is nowhere even a summation sign. On the other hand this emphasizes the adequacy of simple methods for almost all purposes. The index has no place for "hereditary", "familial", or "heredo-familial", and the studious evasion of these confusing words is perhaps more effective as an example than any direct attack on the terminology all too prevalent in medical text-books. Partial sex-linkage is, however, politely quarantined alongside Y-borne genetic disorders.

Radiation is hardly mentioned, but here again, since increasing knowledge has not yet led to an increased consistency of expert opinion, its evasion seems wise. Only three pages are given to twins; none to the even more treacherous subject of the sex ratio.

The author has written little on his particular subjects of the association of blood groups and disease[1] and the inheritance of blood pressure. While this will disappoint most readers, it preserves the balance. Nuclear sexing is mentioned, but not illustrated and barely discussed.

The book was just too early to display the fruits of modern cytological methods, but their technical excellence is illustrated by photomicrographs of the normal chromosome complement.

In such a book lucidity can be achieved only by brevity and even dogma; there are few references, but the author can draw on a personal experience sufficiently extensive to relegate second-hand knowledge to second place. Some statements may, however, cause surprise. The assertions that recessive genes are important in schizophrenia, and a dominant gene in gout, seem strange company for the intervening paragraph on hypertension. Diabetes, kuru, and manic depressive psychosis, the other occasional aspirants to simple genetic explanations, are omitted. Polymorphic and polygenic variation are discussed as being phenomenally, rather than perceptually, different, and related to euchromatin and heterochromatin respectively. Recessively determined achondroplasia is not mentioned in the paragraph relating to the genetic prognosis in this condition. The parents are said to be always affected in the nail-patella syndrome. More boys than girls are said to die in utero.

As in other fields of medicine, the prognosis of the most expert is usually the most optimistic, even if it is only one of authoritarian ignorance, and the final chapter on genetic prognosis explains with simplicity the principles involved, and provides reliable data on the recurrence rate of most of the commoner congenital malformations (excluding congenital heart disease). This is a very useful chapter which does more than fill a void, as a considerable volume of misleading advice is available elsewhere.

This book can be recommended both as a standard work for the clinician and as an introductory work to such specialized subjects as serology and biochemical genetics.

J. H. Edwards

British Pharmaceutical Codex 1959

Pharmaceutical Society of Great Britain. London: Pharmaceutical Press, 1959. xxix + 1301 pages. 23 × 15 cm. £3 10s.

The first *British Pharmaceutical Codex* appeared in 1907, having been prepared by the Pharmaceutical Society of Great Britain as a book of reference for those engaged in prescribing or dispensing medicines. At first, new editions were issued every ten years, but medicine later on advanced so rapidly that new editions were required every five years (and even these lag behind new developments). This edition appeared at the end of 1959, although the recommendations on which it is based were of course made one or more years earlier. The *Codex* is a kind of supplement to the *British Pharmacopoeia*. The *Pharmacopoeia* gives the official legal descriptions of the well-recognized drugs and lays down standards as to their purity and other attributes. The *Codex* has two important functions, namely, to give information (non-official but valuable) on drugs and other pharmaceutical materials, and to provide specifications for those substances which are not included in the *Pharmacopoeia*. Many of these substances are new drugs which have been recently introduced, and there is still insufficient experience to decide whether they should be given legal recognition in the *Pharmacopoeia*. The information given by the *Codex* on the actions and uses of drugs differs from that in most other reference books in that it has been prepared after discussion of the published evidence and private experience by members of a subcommittee of medical and pharmaceutical experts. This applies both to the 70 new monographs in Part I of the present edition, and also to the 667 monographs which were in the 1954 *Codex* or the 1957 *Supplement*, and which have been scrutinized and revised.

The *Codex* is divided into six parts and there are 17 appendices. Part I contains over 800 pages of general monographs describing almost as many medicinal substances. For each substance, the monograph gives the chemical constitution and properties, the solubility and the standard limits of purity; then there is a brief account of its action and uses (including the possible toxic effects) and the dosage and methods of administration, together with details about sterilization and about the preparations which are officially available. The names of the substances and of the preparations are now given in English, although the synonyms usually

[1] See Fraser Roberts, *Brit. med. Bull.* 1959, 15, 129.—Ed.

Book Reviews

The Thyroid Hormones

Rosalind Pitt-Rivers & Jamshed R. Tata, with a chapter on diseases of the thyroid by *W. R. Trotter*. London: Pergamon Press, 1959. (International Series of Monographs on Pure and Applied Biology.) xiii + 247 pages; 22 figures. 23 × 16 cm. £2 10s.

The development of our knowledge of the thyroid gland forms a fascinating study and, although there are many earlier references, it dates principally from the beginning of the nineteenth century. At that time the clinical features of thyrotoxicosis were described, the element iodine was discovered, and the first investigations were made of its importance in the pathogenesis and treatment of goitre. By the end of the century physiological studies had clearly demonstrated the nature of myxoedema, and replacement therapy was introduced. It was again the turn of the chemist and successively there followed the demonstration of the high concentration of iodine in the thyroid gland, the isolation of thyroxine, and finally the elucidation of the structure of this hormone. Subsequently the introduction of chromatographic analysis and the availability of radioactive isotopes of iodine laid the path for further advances. In this way tri-iodothyronine has been demonstrated in the thyroid gland and in the plasma, and the formation, distribution and metabolism of thyroid hormones have been further elucidated. All these physiological and biochemical studies have been described in detail and critically reviewed in this excellent book. Pharmacological aspects have also been considered and there is a full account of the nature and action of substances which inhibit thyroid function.

The authors have provided an extensive review of the published work on their subject, but this book is far from a mere compilation of experimental observations and deductions. The judicious analysis of recent work and the emphasis on the important questions that are at present obscure make stimulating reading. Dr Pitt-Rivers herself played a large part in the isolation of tri-iodothyronine, and it has been suggested that thyroxine had to be de-iodinated to this compound before the typical peripheral effects became apparent. There is, however, a discriminating account of the reasons why this view is untenable and it is suggested that a search for rapidly acting derivatives of thyroxine may have led us in the wrong direction in the elucidation of thyroid hormone action. We are still ignorant of the mechanism by which iodine is concentrated in the thyroid and of the mechanism of action of the hormones, and recent investigations of these difficult topics are fully discussed. Here again the comments made by the authors are pertinent and thought provoking.

The book concludes with a chapter on thyroid diseases written by W. R. Trotter, and this is in keeping with the main theme. Attention is concentrated on the aetiology of various thyroid disorders. In spite of the great advances in the biochemical and physiological aspects, the causation of thyrotoxicosis and of sporadic goitre is still unknown; but there is an impressive review of the relevant evidence.

The authors are to be congratulated on producing a book which is certainly the best account of the subject available at the present time. It is clearly written, attractive in style and only occasionally marred by oversights in proof-reading. No serious investigator in the thyroid field can afford to be without it.

G. M. Wilson

Principles of General Physiology. Volume 1: The Physico-Chemical Background

L. E. Bayliss. 5th ed. London: Longmans, Green & Co. Ltd., 1959. xiii + 520 pages; figures. 24 × 16 cm. £2 15s.

Although this is nominally the fifth edition of Sir William Bayliss's *Principles of general physiology*, the text has been almost completely rewritten by Sir William's son, Dr L. E. Bayliss. The first edition appeared in 1915 and the fourth in 1924. In those editions there were several chapters and parts of chapters which dealt with the basic physical chemistry of biological systems. Since they appeared, however, there have been great changes in physico-chemical ideas, perhaps most significantly in the theory of electrolytes and of dilute solutions. These new ideas have had a profound bearing on physiology, particularly as the processes of biology mostly take place in dilute electrolyte solutions. With these changes in mind, Dr Bayliss thought it best to bring together all the physico-chemical background to biology into a separate first volume, leaving himself a second volume in which to deal more particularly with general physiology itself. This division of the book into two moderately sized parts certainly makes for greater ease in its use, and will doubtless appear less daunting than a single enormous tome.

In writing this book Dr Bayliss has assumed the reader to have an elementary knowledge of physics and chemistry, and to be familiar with elementary mathematics, including a nodding acquaintance with the infinitesimal calculus. The subjects covered include energy and thermodynamics; the kinetics of chemical reactions and their energy changes; the theory of solutions, osmotic effects, viscosity; and weak and strong electrolytes, acids and ampholytes. The section on surface phenomena treats surface energy, surface tension, monomolecular layers, adsorption and surface electrical phenomena. There are also sections on the colloidal state, catalysis and enzymes, oxidation-reduction systems, the actions of light and other radiations, and a final one on the passage of substances through membranes.

Since so wide a field has been covered, it is inevitable that there has been a great deal of compression. Perhaps one of the weaknesses of this volume is the enormous range of subjects dealt with: it might have been better to have left out some topics and to have expanded others. One also gains the impression that, in some sections at least, many concepts are included which have outlived their usefulness. Thus the section on enzyme-catalysed reactions has rather an old-fashioned character, a considerable amount of space being occupied by descriptions of such outmoded ideas as the Schütz rule and chain reactions. Most of the examples quoted in this section are from very old work—not that this necessarily means that they are invalid, but many of the recent significant changes which have occurred in our ideas on enzymes are hardly dealt with.

Despite these criticisms this is an excellent and interesting book, and will prove to be a mine of information and instruction for many biologists. It is not a book for light reading, but it will well repay study and it is written in a manner which enables one to grasp the essential point of the argument easily.

Finally it should be mentioned that at the end of each chapter there are numerous references to reviews and books where further information and inspiration may be found. After reading this volume, one is left impatiently awaiting the publication of the second half.

S. P. Datta

Isotopic Tracers: A Theoretical and Practical Manual for Biological Students and Research Workers

G. E. Francis, W. Mulligan & A. Wormall. 2nd ed. London: Athlone Press, 1959. xxi + 524 pages; 74 figures. 22 × 14 cm. £2 12s. 6d.

Training in isotopic tracer techniques now forms an essential part in the apprenticeship of every biological research worker, and the appearance of a revised and enlarged second edition of this deservedly popular text-book will be especially welcome to all concerned in the application of tracer methods to biochemistry. This volume is essentially a laboratory manual, and contains a wealth of practical information concerning methods of preparation, handling and assay of radioactive materials.

The first part of the book is concerned with theoretical considerations. After a brief introduction, five chapters deal clearly and concisely with the fundamental principles of radioactivity physics, the preparation of radioisotopes and methods of synthesis of labelled compounds. The next two chapters are concerned with general methods for the assay of stable and radioactive isotopes respectively. They are followed by two chapters dealing with the

principles governing the choice of radioactive assay technique in biological investigations and the interpretation of such assay measurements. The relative merits of different assay methods are clearly stated. Subsequent chapters are concerned with autoradiographic techniques, radiation hazards, and examples of the use of isotopic tracer techniques in biological research. Newly added sections include discussions of activation analysis and exchange labelling techniques with tritium.

The second part of the book gives details of 13 exercises comprising a practical course in radioactive isotope techniques. These exercises include the measurement of thyroid activity in terms of the protein-bound radioiodine in the blood after injection of ^{131}I, the localization of injected ^{131}I in the thyroid and the paper chromatography of ^{131}I-containing urines.

Throughout the book, the treatment of the subject is essentially practical. Much valuable and well-balanced advice is given concerning the relative merits of different assay systems. References are limited for the most part to text-books and review articles. It should be emphasized that this volume is written primarily for the laboratory worker and that the chapters dealing with uses of tracer techniques are concerned almost entirely with applications in the biochemical field. Medical diagnostic and therapeutic uses of radioisotopes are only briefly discussed, and physiological and physico-chemical uses receive only cursory mention.

A few minor criticisms may be recorded. The chapter dealing with radiation hazards comprises 30 pages concerned with theoretical aspects of radiation damage and only nine concerned with practical precautions in the use of radioactive materials. In a practical laboratory manual, more detailed information on the design and management of laboratories dealing with radioactive materials would be desirable. Certain of the recommendations which are quoted relating to maximum permissible radiation dose have now been rendered obsolete by the 1958 Recommendations of the International Commission on Radiological Protection. The examples of biological uses of isotopic tracers to study the kinetics of biological processes could be improved by the more liberal use of diagrams. However, exception cannot be taken to a few such deficiencies in a text-book dealing with a rapidly developing field, and this book can be warmly recommended to research workers in the biological sciences as an authoritative introduction to isotopic techniques.

E. H. Belcher

An Introduction to Medical Genetics

J. A. Fraser Roberts. 2nd ed. London: Oxford University Press, 1959. x + 263 pages; 107 figures. 25 × 17 cm. £1 15s.

The task of reviewing this book is difficult because there is no other with which it can be compared. There are excellent books on blood groups, coagulation defects, metabolic disorders, mental deficiency, and so on; there are books introducing the statistician to human data, the medical student to the fruit-fly, and the drosophilist to man. But since the first edition of this book 20 years ago there has been no lucid, sound and detailed book introducing man to man in the field of genetics.

Dr Fraser Roberts covers the main field of human genetics against a background of reliable pedigrees profusely illustrated by photographs and *x* rays. Most of the conditions are necessarily rare, but provide a more satisfactory introduction to medical practitioners, for whom this book is written, than do the conditions in various animals, insects or plants which form the usual background of the non-medical geneticist.

Various deficiencies may be noted by the specialist within the various branches, but even these seem wholly commendable in a book of 250 pages. Mathematical analysis and symbolism are almost absent: there is nowhere even a summation sign. On the other hand this emphasizes the adequacy of simple methods for almost all purposes. The index has no place for "hereditary", "familial", or "heredo-familial", and the studious evasion of these confusing words is perhaps more effective as an example than any direct attack on the terminology all too prevalent in medical text-books. Partial sex-linkage is, however, politely quarantined alongside Y-borne genetic disorders.

Radiation is hardly mentioned, but here again, since increasing knowledge has not yet led to an increased consistency of expert opinion, its evasion seems wise. Only three pages are given to twins; none to the even more treacherous subject of the sex ratio.

The author has written little on his particular subjects of the association of blood groups and disease[1] and the inheritance of blood pressure. While this will disappoint most readers, it preserves the balance. Nuclear sexing is mentioned, but not illustrated and barely discussed.

The book was just too early to display the fruits of modern cytological methods, but their technical excellence is illustrated by photomicrographs of the normal chromosome complement.

In such a book lucidity can be achieved only by brevity and even dogma; there are few references, but the author can draw on a personal experience sufficiently extensive to relegate second-hand knowledge to second place. Some statements may, however, cause surprise. The assertions that recessive genes are important in schizophrenia, and a dominant gene in gout, seem strange company for the intervening paragraph on hypertension. Diabetes, kuru, and manic depressive psychosis, the other occasional aspirants to simple genetic explanations, are omitted. Polymorphic and polygenic variation are discussed as being phenomenally, rather than perceptually, different, and related to euchromatin and heterochromatin respectively. Recessively determined achondroplasia is not mentioned in the paragraph relating to the genetic prognosis in this condition. The parents are said to be always affected in the nail-patella syndrome. More boys than girls are said to die in utero.

As in other fields of medicine, the prognosis of the most expert is usually the most optimistic, even if it is only one of authoritarian ignorance, and the final chapter on genetic prognosis explains with simplicity the principles involved, and provides reliable data on the recurrence rate of most of the commoner congenital malformations (excluding congenital heart disease). This is a very useful chapter which does more than fill a void, as a considerable volume of misleading advice is available elsewhere.

This book can be recommended both as a standard work for the clinician and as an introductory work to such specialized subjects as serology and biochemical genetics.

J. H. Edwards

British Pharmaceutical Codex 1959

Pharmaceutical Society of Great Britain. London: Pharmaceutical Press, 1959. xxix + 1301 pages. 23 × 15 cm. £3 10s.

The first *British Pharmaceutical Codex* appeared in 1907, having been prepared by the Pharmaceutical Society of Great Britain as a book of reference for those engaged in prescribing or dispensing medicines. At first, new editions were issued every ten years, but medicine later on advanced so rapidly that new editions were required every five years (and even these lag behind new developments). This edition appeared at the end of 1959, although the recommendations on which it is based were of course made one or more years earlier. The *Codex* is a kind of supplement to the *British Pharmacopoeia*. The *Pharmacopoeia* gives the official legal descriptions of the well-recognized drugs and lays down standards as to their purity and other attributes. The *Codex* has two important functions, namely, to give information (non-official but valuable) on drugs and other pharmaceutical materials, and to provide specifications for those substances which are not included in the *Pharmacopoeia*. Many of these substances are new drugs which have been recently introduced, and there is still insufficient experience to decide whether they should be given legal recognition in the *Pharmacopoeia*. The information given by the *Codex* on the actions and uses of drugs differs from that in most other reference books in that it has been prepared after discussion of the published evidence and private experience by members of a subcommittee of medical and pharmaceutical experts. This applies both to the 70 new monographs in Part I of the present edition, and also to the 667 monographs which were in the 1954 *Codex* or the 1957 *Supplement*, and which have been scrutinized and revised.

The *Codex* is divided into six parts and there are 17 appendices. Part I contains over 800 pages of general monographs describing almost as many medicinal substances. For each substance, the monograph gives the chemical constitution and properties, the solubility and the standard limits of purity; then there is a brief account of its action and uses (including the possible toxic effects) and the dosage and methods of administration, together with details about sterilization and about the preparations which are officially available. The names of the substances and of the preparations are now given in English, although the synonyms usually

[1] See Fraser Roberts, *Brit. med. Bull.* 1959, **15**, 129.—ED.

include the Latin equivalent. Part II (which occupies 50 pages) deals with antisera, vaccines, and related products. This section includes a monograph on poliomyelitis vaccine which will be consulted with interest, although the most important items, viz. the standardization of safety and antigenicity, are covered by the cryptic statement: "This vaccine must comply with the requirements of the Licensing Authority", i.e., the Ministry of Health. A new monograph has also been introduced on rabies antiserum. Part III (14 pages) describes preparations of human blood such as whole blood, fibrinogen, plasma. Part IV (8 pages) deals with surgical ligatures and sutures, and it has been considerably expanded by the addition of a general monograph on non-absorbable sutures and of ten monographs describing five non-sterile and five sterile sutures. Part V (38 pages) deals with surgical dressings and it contains nothing new, but penicillin gauze has been omitted because it may cause sensitization. Part VI (224 pages) is the Formulary which gives methods of preparations and specifications for the various extracts, infusions, ointments and other pharmaceutical preparations. New formulae are included only if the substances named in them are freely available to pharmacists; since most new drugs are not so available, being supplied only as the products of a single manufacturer, they are not included in this section. The 17 appendices, which occupy 68 pages, give atomic weights, details of reagents and methods of carrying out the most important tests to check composition, sterility, and toxicity.

The *Codex* is now such an established authority on the production, handling and dispensing of drugs and medicinal substances that it needs no recommendation from a reviewer—just as good wine needs no bush. One can only congratulate all those who have taken part in its preparation for having compiled a volume which is fully equal to the high standard set by the preceding editions.

F. Hawking

Pharmacopoea Internationalis, Supplementum

World Health Organization. Geneva: World Health Organization, 1959. xx + 224 pages. 25 × 16 cm. £1 5s.

The International Pharmacopoeia has been produced as a collection of recommended specifications regarding drugs and medicinal products. It is not intended to have legal status as such in any country (the "world state" has not yet arrived), but its specifications are offered to serve as references for any country which wishes to establish its own specifications. In fact, the specifications of the International Pharmacopoeia are being used to an increasing extent as a practical guide in the drawing up of standards for pharmaceutical preparations in many different states.

Any member state of the World Health Organization (WHO) may include a translation of all or part of these provisions in its national pharmacopoeia, and WHO will grant the rights to publish a translation of the International Pharmacopoeia when desirable. Up to date, the volumes have been issued in English, French and Spanish, and they have been translated into German and Japanese.

The present supplement is designed to complete the information which has already been given in volumes I and II. It contains a further 94 monographs on medicinal substances, and 17 appendices. These have been prepared by various WHO expert groups convened in Geneva during the period 1956–58; then the specifications have been submitted to the member states of WHO, and the text has been revised after discussion of the comments received. The monographs deal with a great variety of medicinal substances, e.g., hormone preparations (dienoestrol, hydrocortisone, methandriol and insulin zinc), antimalarial compounds (chloroquine, primaquine and pyrimethamine), contrast media (acetrizoic and iopanoic acids) and certain antibiotics (erythromycin, polymyxin B and tetracycline). Each monograph gives the formula, a description of the physical state of the substance, the solubility, methods for identification and for assay, and notes about storage. Nothing is said about uses and toxicity, but the usual, and the maximal, doses for adults are given in an appendix (no. 14). The appendices describe reagents and test solutions, and methods that may be needed to check the purity and identity of the medicinal substances mentioned in the monographs. A special appendix (no. 7) gives graphs showing the amount of sodium chloride which must be added to solutions of 67 different substances in order to make them isotonic with human tissue and blood. Other appendices give methods for determining calcium, zinc and the potencies of erythromycin and polymyxin. Appendix 16 gives an up-to-date list headed "International Biological Standards and International Biological Reference Preparations". An annex gives a few small amendments to volumes I and II of the International Pharmacopoeia.

By its very nature, the International Pharmacopoeia and its supplements are volumes for the experts who will draw up their own national pharmacopoeias rather than for the practising pharmacist who will ultimately have to observe them. But since these volumes will lead to an increasing standardization of pharmaceutical practice throughout the world (and it is hoped that this standardization will take place at the high levels of the more fully developed countries), they are to be welcomed and supported as yet another embryonic structure of the Utopian "world state". If the nations cannot agree on measures of disarmament, at least let them agree about the activity of insulin preparations and of antibiotics. Perhaps the habit of agreeing on minor things may lead to a greater willingness to agree on major things also.

F. Hawking

Doctors' Commons: A Short History of the British Medical Association

Paul Vaughan. London: William Heinemann Ltd., 1959. xvi + 254 pages; 17 plates; 7 illustrations. 22 × 15 cm. 18s.

It is a far cry from the board-room of the Worcester Infirmary in 1832 to BMA House, Tavistock Square, London, in 1960. In the former "more than fifty medical gentlemen" met to form the Provincial Medical and Surgical Association. In the latter are housed the head-quarters of the British Medical Association, whose membership exceeds 73,000 and whose income last year was £310,000. This transformation has not been achieved without many vicissitudes. The Association may have changed its names, but its members have never changed their innate objection to regimentation. The founder of the Association, Sir Charles Hastings, was soon to discover that the British doctor is a disputatious animal with all the traditional obstinacy of the mule if he thinks that he is being forced into a common mould.

The reasons for this are not far to seek. He is first and foremost an individualist. Secondly he is an empiricist. Thirdly he distrusts logic because he knows that human beings are seldom logical. Fourthly he puts his duty to his patient before everything else, which means that he cannot allow himself to be bound by any rules outside the Hippocratic Oath. To attempt to organize such a team is obviously asking for trouble, as the leaders of the Association have found from its earliest days. Indeed, the wonder is that the British Medical Association has achieved as much as it has done in organizing the profession.

The story of this achievement is admirably recounted by Mr Paul Vaughan, the Chief Press Officer of the Association. He may at times have donned his rose-tinted spectacles, but on the whole he is reasonably objective, and his sense of humour prevents his ever becoming ponderous. This is in no sense an official history. Neither, let it be added, is it a profound one, but it is one that can be recommended to the reader who wishes to obtain an over-all picture of the activities of the Association from its beginnings. Written in a pleasant journalistic style, which makes for easy reading, it depicts the general trend of development of the Association with admirable clarity. This in itself is a commendable feat. As more than one historian of the Association has found to his cost, it is dangerously easy to get so befogged with detail that the reader is unable to see the wood for the trees. Mr Vaughan has successfully avoided this danger. His is a composite picture in which detail has been blended into a swiftly moving panorama covering one of the most momentous eras in British medicine.

William A. R. Thomson

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Printed in Great Britain by Spottiswoode, Ballantyne & Co. Ltd., London & Colchester

Scientific Editor: *F. G. Young*

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Insulin and Diabetes

Medical research ranges over ever widening fields of endeavour, and the development of our knowledge of diabetes and of its therapeutic agent, insulin—the most economically important of all purified proteins—well illustrates this fact. The date of the first description of diabetes is ancient but unknown, though claims have been made on behalf of China, India, and Egypt as home of the discoverer of this wasting disease. Attempts were early made to control the symptoms of diabetes by dietary variation and restriction but these were generally ineffective and the condition in its severe form remained an ultimately fatal disease until the present century. By the eighteenth century diabetes, the most striking symptom of which was polyuria, was divided into two types, one in which the urine tasted sweet—diabetes mellitus—and the other in which the sweet taste of the urine was lacking—diabetes insipidus. This discovery naturally focused attention on the biological significance of sugars in the study of diabetes mellitus.

The fundamental importance of the researches of Claude Bernard into the metabolism of carbohydrate is clear, yet his investigations did not reveal the cause of diabetes mellitus nor improve the treatment of this condition. The controversies between Bernard, Pavy, and others

about the mechanism of control of the amount of sugar in the blood and the aetiology of diabetes[1] were interesting but not of direct value in preserving the patient with a severe form of the disease. The so-called "piqûre diabetes" of Bernard was little more than a transient glycosuria seen in a well-fed animal, and its fame tended to direct interest away from consideration of a hormonal origin of diabetes to obscuring ideas about the possible significance of an excessive stimulation of secretory nerves to the liver as the cause of the rise of the blood-sugar level seen in this condition. In the nineteenth century the controversies between those who considered the nervous system to be of primary significance in the co-ordination of metabolic activities, and those who asserted the importance of humoral agents in this respect, were more productive of polemics than of adequate evidence for the views asserted.

Until a persisting condition closely resembling natural diabetes mellitus could be induced in animals the cause of the clinical condition was largely a matter for conjecture. The accidental discovery in 1889 by von Mering and Minkowski of the diabetes-inducing effect of removal of the pancreas was itself the result of argument about the possible dietary value of partially hydrolysed fat[2], but fortunately those concerned in these interchanges believed in experiment as well as in discussion. At once a field of investigation was opened for exploration which had been quite unknown to Claude Bernard.

The observation in 1901 by Opie that the islets of Langerhans of the pancreas are sometimes abnormal in appearance in human diabetes mellitus provided ancillary evidence for the belief that diabetes could result from a defect in the pancreas, but attempts to prepare an extract of this organ which, on parenteral administration, would alleviate the symptoms of diabetes mellitus—both natural and experimental—were unsuccessful for many years. Some clinicians, for example Zuelzer[3], possibly did succeed in preparing extracts of the pancreas which contained insulin, but these sometimes produced alarming reactions in treated patients, perhaps because an overdose of the hormone had been administered and the blood sugar had temporarily fallen to a dangerously low level. The importance of using animals and not patients in the early stages of an investigation, and the value of the development of micromethods which permitted frequent estimation of the amount of sugar in the blood, are both emphasized by these experiences.

The careful experiments of Frederick M. Allen[4] with partially depancreatized dogs led to well-founded advances in the dietary alleviation of the more serious symptoms of human diabetes mellitus, while others used a diet rich in fat in the treatment of this condition, notably Newburgh and Marsh[5] and Petrén[6]. But the use of insulin ultimately turned attention away from the possible dietary control of diabetes mellitus, perhaps to an undesirable extent.

So confident were de Meyer[7] and Schäfer[8] that the islets of Langerhans secreted a hormone a deficiency of which could result in diabetes mellitus, that the then unborn hormone was named "insuline". Such prenatal invention of a surviving almost unaltered name is probably unique and when in 1921 Banting and Best at last brought the elusive substance into the light of day the appropriateness of the name with only a slight emendation was not long in doubt.

That insulin is not yet forty years old is perhaps surprising to those to whom its place in medicine seems to be as well established as that of anaesthetics or of aseptic surgery. Yet C. H. Best, one of the two co-discoverers of insulin, is still vigorously active in advancing medical knowledge and contributes the first paper to this number of the *British Medical Bulletin*. The light that he throws on many incidents in the early days of his researches with Banting is of interest and importance to historians of medicine as well as to many others.

Once insulin had been isolated and characterized as a protein—it was first obtained in crystalline form by Abel in 1926—its chemistry naturally attracted wide interest. To many it is particularly pleasing that in these days when the importance of individual, as opposed to group, activity is sometimes in danger of neglect, the complete chemical structure of insulin should have been elucidated not by a large "team" of research workers "headed" by somebody but by one who may reasonably be described as a modest individualist—Frederick Sanger, whose researches were pursued in co-operation with no more than one or two junior colleagues at a time. That this investigation should have been brought to a successful conclusion within a period of about ten years (1945–55)

[1] See Young, F. G. (1937) *Ann. Sci.* **2**, 47; (1957) *Brit. med. J.* **1**, 1431
[2] See Houssay, B. A. (1952) *Diabetes*, **1**, 112
[3] Zuelzer, G. L. (1908) *Z. exp. Path. Ther.* **5**, 307
[4] Allen, F. M. (1913) *Studies concerning glycosuria and diabetes*. Harvard University Press, Boston; Allen, F. M., Stillman, E. & Fitz, R. (1919) *Total dietary regulation in the treatment of diabetes*. (*Monographs of the Rockefeller Institute for Medical Research*, No. 11.)
[5] Newburgh, L. H. & Marsh, P. L. (1920) *Arch. intern. Med.* **26**, 647
[6] Petrén, K. (1924) *J. metab. Res.* **5**, 7
[7] Meyer, J. de (1909) *Arch. Fisiol.* **7**, 96
[8] Schäfer, E. A. (1916) *The endocrine organs*, p. 128. Longmans Green, London

is truly astonishing and a great tribute to Sanger's foresight, persistence, and experimental ability. This research involved an immense amount of painstaking manipulative work at the laboratory bench, the plan of which rested upon hard logical thinking with careful preliminary consideration of experimental possibilities and subsequent critical interpretation of results. The award of the Nobel Prize for Chemistry for 1958 to Sanger was a fitting conclusion to an outstandingly fruitful period of research and the publication is appropriate in this number of the Bulletin of a slightly expanded version of the lecture which Sanger gave on the presentation of the Nobel Prize.

Although knowledge of the structure of insulin does not in itself lead to an understanding of its action, nor modify the use in practice of this valuable therapeutic agent, one can say with certainty that the more that is known about the structure of a substance the more likely it is that better methods can be developed for its preparation. Moreover, once the chemical structure of anything is known its synthesis in the laboratory becomes at least a possibility, though many years must elapse before chemically synthesized insulin is cheaply available. Whether indeed synthetic insulin will ever be able to compete in price with insulin extracted from natural sources is at least questionable. Nevertheless the number of diabetic patients in the world rises steadily and the supply of insulin from natural sources is not unlimited, so one must be prepared for the possibility that ultimately synthetically prepared insulin or related substances will be essential. Sanger's chemical researches on insulin have guided the world's first steps along a path which is of immense theoretical significance and potentially of great practical importance.

From his investigations has emerged the chemical basis of the species differences which have long been suspected in insulin, and in his contribution to this number of the Bulletin Sanger reports recent studies which have revealed the structure of insulin from the human pancreas. The human hormone is now known to differ from the commercially available insulins but most closely resembles that from the pig, and the value of insulin from pig pancreas in patients in whom reactions of an allergic type have been encountered with other types of insulin has long been recognized.

Biological activity does not appear to depend upon the integrity of the insulin molecule as a whole, and the same is true for certain other peptide hormones, for example corticotropin from the anterior pituitary gland. A peptide hormone, and indeed any other biologically active peptide, may carry some part of its peptide chain as a sort of protective coat, not needed for biological activity but valuable for guarding the more active parts of the molecule against adventitious degradation.

The variety of the methods of assay of insulin which are now available is almost bewildering, though of course it is not certain that different types of assay depend upon the same chemical structures in the molecule. The effective clinical use of insulin depends vitally upon the accuracy of the biological standardization of the manufactured product, and G. A. Stewart's paper sets out clearly the advantages and limitations of the multiplicity of methods which are now available for routine assay and for research purposes.

Although the clinical usefulness of insulin relates to the accuracy of its biological assay, its clinical value fortunately in no way depends upon an understanding of its mechanism of action. Indeed, when one considers the multitude of scientific papers that have been published since the discovery of insulin forty years ago, the uncertainty of knowledge about the mechanism of action of this hormone is surprising. Whether or not this substance has a single point of action or acts upon different processes is far from clear at the present time. The fact that insulin acts on carbohydrate metabolism is clearly shown by its potent effect in abolishing hyperglycaemia and glycosuria in diabetes and by the rapid fall of blood-sugar level which follows its administration to normal animals or man. The actions of insulin upon fat metabolism and upon protein metabolism are perhaps no less important though less obvious, but now they are being subjected to detailed examination.

The fact that a hormone can act in a physiologically significant way by antagonizing the action of other hormones was firmly established by the investigations of B. A. Houssay and his colleagues in Argentina in the late 1920's and early 1930's. But the antagonists to insulin have multiplied in number out of all reasonable expectation in recent years and the modern picture is indeed a complicated one. It has been said that insulin with its potent hypoglycaemic action is like an enormously strong Gulliver held down by many Lilliputian insulin antagonists whose task is to keep the giant under close control lest his vigorous activity wreck the delicacy of the whole structure. Be that as it may, there is now clear

evidence that a balanced antagonism between substances of physiological importance but with opposing actions provides a delicate means of control of biological processes.

We know remarkably little about the mechanism of control of secretion of insulin, although the enhanced rate of liberation of this hormone into the blood when the blood-sugar level rises is now well recognized. We know even less about the biosynthesis of insulin in the pancreatic islets and the control of this important function. And what goes wrong with insulin production in diabetes is still wrapped in darkness which perhaps electron microscopy may help to illuminate. The precise nature of the insulin-active material which circulates in the blood remains another matter for future research.

The fact that substances of a comparatively simple chemical nature and entirely different from insulin may have an action resembling that of this hormone in the diabetic patient has been realized since a few years after insulin itself was prepared, although some of the early claims that simple chemical substances could exert a significant hypoglycaemic action have not subsequently been substantiated. The modern era, with its emphasis on the hypoglycaemic activity of sulphonamide derivatives given by mouth, may be said to date from the important experiments of Loubatières and his colleagues at Montpellier, France, in 1942, which arose themselves from the clinical observations of Janbon. In 1942 Janbon and his collaborators at Montpellier observed that hypoglycaemia sometimes developed when patients with typhoid fever were treated with *p*-aminobenzenesulphonamido*iso*propylthiodiazole, and that under the dietary conditions which obtained in France at that time this hypoglycaemia was sometimes fatal. It was this observation by his clinical colleagues that Professor Auguste Loubatières followed up, and as a result was able to show convincingly that a number of sulphonamide derivatives given by intravenous means or by mouth could produce a profound hypoglycaemia in animals and in patients. In 1955 a number of German investigators described the hypoglycaemic and antidiabetic action of the oral administration of a different series of sulphonamides, and subsequently many other substances, related and unrelated, have been examined for possible clinical use in diabetes mellitus.

On the balance of the evidence at present available it seems likely that the action of these synthetic substances depends on the presence of small amounts of insulin in the body and that in total diabetes they are ineffective. But their mechanism of action is still obscure although, as will be seen from the paper by J. D. N. Nabarro which closes this number of the Bulletin, they are widely and effectively used in the treatment of some types of diabetes mellitus.

Growth of knowledge about the biological action of insulin was for many years more rapid than that about its chemical structure. Researches on its chemistry have now, perhaps temporarily, outpaced others. Nevertheless we can be sure that the elusiveness, importance, and subtlety of action of insulin will continue to attract investigations on all aspects of this hormone and its effects for many years to come.

F. G. Young

We are indebted to Professor F. G. Young for the help he gave in acting both as Chairman of the planning committee for this number and as Scientific Editor.

MEDICAL AND SCIENTIFIC SECTION OF THE BRITISH DIABETIC ASSOCIATION

The British Diabetic Association was founded in 1934. The principal objects of the Medical and Scientific Section are (i) to promote the study of the causes and treatment of diabetes and associated diseases; (ii) to help to diffuse among doctors and scientific workers knowledge of the scientific and clinical aspects of diabetes and associated diseases; (iii) to encourage and support research into diabetes and associated diseases. It is open to any suitable qualified person and application forms are available on request.

The Association is affiliated to the International Diabetes Federation, whose headquarters are at 152 Harley Street, London, W.1. This is a federation of 29 national diabetic associations throughout the world. A Congress is organized through the Federation every three years, and the next one will be held on the 10–14 July 1961 in Geneva. Further information will be sent on application to the above address.

FORTY YEARS OF INTEREST IN INSULIN

CHARLES H. BEST C.B.E. M.A. M.D. D.Sc. F.R.S.

Director, Banting and Best Department of Medical Research

Head of Department of Physiology University of Toronto, Canada

1 Early work
2 First publications
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5 Death of Banting
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It will be forty years this autumn since I began to familiarize myself with some of the biochemical and physiological methods which were used to establish the presence of insulin in pancreas.

1. Early Work

In the final year of an honours course in physiology and biochemistry at the University of Toronto, designed to prepare students for a career in research, I applied Dakin's butanol fractionation of amino acids to hydrolysates of the protein gliadin and studied the effects of Claude Bernard's piqûre in turtles. I became familiar with the accepted methods for estimation of sugar in blood and urine, of glycogen, nitrogen, ketone bodies, and of oxygen and carbon dioxide in expired air. The determination that was to be of the greatest use to Banting and me was that of sugar in blood. The Myers-Bailey modification of the Lewis-Benedict procedure was followed and the results were checked against the values obtained by the Shaffer-Hartmann method.

Some notes which another graduating student, E. C. Noble, and I made on the results of our piqûre operations on turtles are in the first note-book which Banting and I used. These experiments were continued after the beginning of my work with Banting on the 17 May 1921. The last entry was on the 16 June of that year. The normal blood sugar of our turtle was 0.08%. Puncture in the area of the "sugar centre" was made at 11.00 a.m. and the blood sugar rose rather rapidly to reach a plateau at 1.55 p.m. Thus ended my first research and the voice of the turtle was heard no more in the laboratory.

In May and June Banting and I ligated the pancreatic ducts in a series of dogs and depancreatized others. The chemical findings were those which we anticipated. In July and August we watched our extracts of degenerated pancreas produce dramatic lowering of the blood sugar of diabetic dogs and improvement in the condition of the animals. On the 14 August 1921 we saw, for the first time, hypoglycaemia develop in a depancreatized dog, following the intravenous injection of 30 ml. of our extract. In November, we lowered the blood sugar of a dog from a very high level (0.40%) to one at which some signs of hypoglycaemia were produced, and we studied the effect of giving glucose. A depancreatized dog was kept alive for 70 days on extracts made from foetal calf and adult beef pancreas.

The small-scale method used from the end of July 1921 for making potent lots of insulin consisted, essentially, of a neutral, or preferably an acid, extraction of the pancreas, using at first saline and later ethanol at about 60% concentration. The cold mixture was filtered and the filtrate evaporated to dryness, either in a small vacuum still or in a rapid current of warm air. The residue was washed with a fat solvent and dissolved in water or saline. This procedure always gave us a potent extract from degenerated dog pancreas, normal dog pancreas, foetal calf pancreas and adult beef pancreas and it was one to which we returned when more complicated methods gave trouble. The insulin used on the first human diabetic subject on the 11 January 1922 was extracted in this way. The method yielded a clear, potent material from foetal calf pancreas which we now know contained about 12 International Units (i.u.)/ml. It passed more easily through a Berkefeld filter than did extracts from a less potent source. This was the material which I wished to use in the first clinical case, but Fred Banting thought that we should make it from commercially available glands. When prepared from adult beef pancreas, the potency of the solution is about one-tenth that of the foetal pancreas extract, and a correspondingly large amount of inert material is present. Recently, my colleagues, Salter, Sirek and Leibel (unpublished data), have shown that an extract of foetal calf pancreas, made as I prepared it in the autumn of 1921, is a very effective non-irritating antidiabetic preparation when given subcutaneously to diabetic children or adults.

In the autumn of 1921, anxious to complete a research for my Master of Arts thesis in physiology, I selected as a problem the effect of insulin on the respiratory quotient (R.Q.) of diabetic dogs and of a diabetic man, Dr Joseph Gilchrist. There were many trials and tribulations later in that academic year. Some of the diabetics who had been started on treatment died from lack of insulin, and of course the number of patients demanding the new remedy increased by leaps and bounds. I found myself forced to return to the large-scale production of insulin, with little time to work on the effect of insulin on R.Q. I felt unable to finish my Master's thesis, but the Head of the Department, Professor J. J. R. Macleod, assured me that the record of almost any definite advance would be acceptable. I depancreatized the dogs, organized the experiments, and collected the expired air in Douglas bags. Dr John Hepburn was working in the Department on another problem and he was good enough to do most of the gas analysis work. The R.Q. of the untreated animals and of Dr Gilchrist were always in the neighbourhood of 0.73; after insulin there was a very definite rise in the ratio. I remember hurriedly preparing a brief manuscript just before the dead-line for the acceptance of my M.A. thesis. I was not very proud of it but the report did contain the first data indicating increased combustion of sugar after the administration of insulin. Final evidence had to wait until isotopically labelled dextrose became available.

2. First Publications

The first paper on insulin was prepared by Banting and myself and was presented to the Medical Faculty and students

on the 14 November 1921. The charts were prepared by Banting, and the original manuscript, which has been preserved, is in large part in my own handwriting. After the paper had been presented, it was read by Professor Macleod, who suggested no changes, and it was immediately submitted for publication (Banting & Best, 1922a). A summary of this paper with Professor Macleod's name appended was presented in late December 1921, at the Thirty-Fourth Annual Meeting of the American Physiological Society, at New Haven, Connecticut (Banting, Best & Macleod, 1922).

Banting and I reported that intravenous injections of extract of dog pancreas, removed 7–10 weeks after the ligation of the pancreatic ducts, invariably decreased the sugar in the blood and the amount of sugar excreted. We noted that the extent and duration of the decrease varied directly with the amount of extract injected, that extract given by the rectal route was not effective and that boiled extract failed to lower blood sugar. We now know that insulin at acid reaction can withstand considerable heat, but prolonged boiling certainly inactivated those early extracts. Pancreatic juice destroyed the active principle. Extracts prepared in neutral saline and kept in the cold retained their potency for at least a week, but the activity disappeared from alkaline extracts. The lowering of blood sugar was not a dilution phenomenon, as indicated by the facts that (i) haemoglobin estimations before and after the administration of the extracts were identical; (ii) injections of large quantities of saline did not affect the blood sugar; and (iii) similar quantities of extracts of other tissues did not cause a decrease in blood sugar. An extract made with 0.1% acid in saline was effective. Injection of the extracts enabled a diabetic animal to retain a much greater percentage of sugar than it otherwise would.

On the 7 February 1922, Banting and I presented our findings at a large meeting of the Academy of Medicine of Toronto (Banting & Best, 1922c). We showed lantern slides to illustrate all the conclusions to be published in our first paper. We reported that foetal calf pancreas of under five months' development did not contain pancreatic enzymes but did provide insulin in very potent form. By macerating the foetal gland in Ringer's solution and filtering it, a clear solution was obtained. To estimate the potency of the extract, we macerated 50 g. of foetal calf pancreas in 250 ml. of acid saline, added a 15-ml. portion of the filtrate to 250 ml. of saline, and injected 15 ml. of this solution. The blood sugar in a 10-kg. diabetic dog was reduced from 0.40% to 0.15% in three hours. We found that this extract, which probably contained 3–5 i.u. of insulin, administered subcutaneously, gave a slower and more prolonged, but not less marked, fall in the blood sugar than the same amount given intravenously. We noted that the potency of our watery extracts could be preserved by the use of tricresol. In the same paper (Banting & Best, 1922c) we reported obtaining

> . . . from [adult beef pancreas] an extract of the active principle which, when washed with toluol, gave a brownish powder which could be kept sterile, which was soluble in saline and which, in minute doses (50 mgms.) gave a pronounced effect on the sugar of the blood.

The third paper on insulin published by Banting and myself (Banting & Best, 1922b) gives further details of the effects of extracts from foetal pancreas.

At the May 1922 meeting of the Royal Society of Canada, a series of papers on insulin were presented, confirming and extending the earlier findings. It was demonstrated that insulin abolished ketosis, prevented deposition of excess fat and stimulated the formation of glycogen in the liver of diabetic dogs (Banting, Best, Collip, Hepburn, Macleod & Noble, 1922).

The first publication on the clinical effects of insulin was a preliminary report (Banting, Best, Collip, Campbell & Fletcher, 1922) which has frequently been overlooked. It was reported that, in severely diabetic patients, insulin (i) reduced the blood sugar to normal values; (ii) abolished glucosuria; (iii) caused acetone bodies to disappear from the urine; (iv) raised the R.Q.; (v) produced a definite clinical improvement and a subjective feeling of well-being and increased vigour. Our findings in diabetic dogs were completely confirmed in human diabetics.

3. Early Manufacture of Insulin

The spring and summer of 1922 was a hectic period in my career. I found myself in charge of the large-scale production of insulin for Canada, having been appointed Director of the Insulin Division of the Connaught Laboratories, Toronto, in January 1922. When I actually assumed full responsibility a few weeks later, no insulin was being produced by Dr J. B. Collip's excellent small-scale procedure, which he had adapted for the production of larger amounts of the hormone. Dr Collip had returned to his Chair of Biochemistry in Alberta. Tests indicated that exposure of insulin to a high temperature during the long distillation was responsible for the loss. We achieved success only when we went back to the same technique that Banting and I had used originally for making extracts of normal beef pancreas, i.e., rapid evaporation of the alcoholic filtrates in a current of warm air. Doing this on a small scale in a laboratory was one thing, but on a scale large enough to provide insulin for scores of patients, was quite another. The use of acetone instead of ethanol for extraction was an advantage because the speed of evaporation of the liquid from the large shallow trays in the wind tunnels was very considerably increased. As soon as the liquid had been blown off it was essential quickly to remove the trays, scrape off the resinous residue, and proceed with its purification. We made many good lots of insulin in the spring and early summer of 1922 by this method. At the same time we were attempting to help Eli Lilly and Company to get started in their programme, this company having been selected by our Insulin Committee as the first pharmaceutical company in the United States of America to make insulin. I made many trips to Indianapolis, and Dr G. H. A. Clowes and his colleagues made more to Toronto. In the early summer of 1922 a 25-gallon vacuum still replaced the cumbersome and dangerous wind tunnel. The chemical engineers of Eli Lilly and Company helped us in many other ways to make insulin available to Canadian diabetics.

Dr D. A. Scott had joined me in the spring of 1922 and rapidly mastered the details of problems which were completely new to him. During the next three years, Dr Scott and I published papers on the preparation of insulin (Best & Scott, 1923; Scott & Best, 1925), including bulletins on the methods for producing insulin on a large scale, which were sent at frequent intervals to interested manufacturers in many parts of the world. Dr Scott's brilliant work on the role of zinc in the crystallization of insulin (Scott, 1934) and, with Dr A. M. Fisher (Scott & Fisher, 1936), in the preparation of protamine zinc insulin, is known to all students of these subjects.

4. Further Developments

I obtained my medical degree in 1925 and soon after went to work with H. H. Dale at the National Institute for Medical Research in London. The first paper on insulin which I published from that laboratory (Best, 1926) provided evidence that insulin greatly accelerates the rate of sugar disappearance from defibrinated blood used to perfuse the isolated limbs of the cat. The next paper (Best, Hoet & Marks, 1926), an extension of the study of Burn & Dale (1924), showed that the disappearance of inorganic phosphate from the blood of the eviscerated spinal preparation after insulin was related to the utilization of sugar and not merely to the fall in sugar concentration of the blood. The disappearance of muscle glycogen in normal rabbits, produced by large doses of insulin, was clearly shown to be due to the convulsions associated with low blood sugar.

Later, using the eviscerated spinal preparation (Best, Dale, Hoet & Marks, 1926), we determined the oxygen consumed and found that the glucose which disappeared under the action of insulin was equal to the sum of the glycogen deposited in the muscles and the glucose equivalent of the oxygen absorbed. This balance was preserved whether the blood sugar was maintained at a high level by rapid infusion of glucose or allowed to sink to a very low level by restricting the supply.

After returning to Toronto as Head of the Department of Applied Physiology in the School of Hygiene, I wrote a long paper on the preparation and standardization of insulin (Best, 1927).

In 1929 I succeeded Professor Macleod as Head of the Department of Physiology and became very much occupied with research work on heparin as an antithrombotic agent. It was during this period that the original demonstration that choline is an important dietary factor in the protection of the liver was made and that Scott and Fisher developed protamine zinc insulin in the Division of the Connaught Laboratories for which I had responsibility.

When Professor F. G. Young's papers on the production of permanent diabetes in dogs by anterior pituitary extracts appeared (Young, 1937, 1938), my colleagues and I were tremendously stimulated by his findings. Best, Campbell & Haist (1939) soon showed that the administration of similar extracts produced a prompt and extensive fall in the insulin extractable from dog pancreas.

In this same period, Dr Haist, Dr Jessie Ridout and I began our work on the effect of diet on the insulin content of pancreas. A long series of experiments in this field have occupied a great deal of Professor Haist's time and made him an authority on the dietary and endocrine factors which control the growth of the islets of Langerhans and their content of insulin. Dr Campbell, whose contributions are equally widely known, interested himself in the pituitary factors which cause mobilization of fat from the depots to the liver, and in detailed observations on the effects of somatotropin and its interrelationships with insulin and other hormones.

Dr Haist, Dr Campbell and I were interested in the prevention, by the administration of insulin, of diabetes induced by anterior pituitary extracts in dogs (Haist, Campbell & Best, 1940; Haist & Best, 1941). We suggested that insulin might be given to children in whom it appeared almost certain that diabetes would develop. This suggestion has never been adequately followed up for the simple reason that the psychological trauma of the injection has, probably quite rightly, been considered to have more potentialities for harm than the use of insulin would have for good. This is interesting in view of the modern researches in which oral hypoglycaemic agents, which may liberate some pancreatic insulin, are being tested for their value in the prevention of diabetes.

When the threat of war appeared, our studies on insulin, choline and heparin were abandoned to make way for the preparation of dried human blood serum, for investigations on traumatic shock, and later for a wide range of other pressing military medical problems.

5. Death of Banting

It is difficult to describe the sense of personal loss produced by Frederick Grant Banting's death on the 21 February 1941, while on active military service. I may quote from an obituary notice which I wrote for the Royal Society (Best, 1942):

> The year 1921 was certainly one of the most strenuous and the most productive of Banting's life. He demonstrated at that time four great traits of his character—courage, persistence, scientific ingenuity and industry. . . The memory of the intimate association with Banting during that spring, summer and autumn, when it became clear that an active diabetic principle could be prepared from degenerated or from normal pancreas, will always be cherished. The intense excitement and pleasure with which we watched the depth of colour in the sugar reagent fade as the blood sugars of the diabetic animals became reduced under the action of insulin, is difficult to describe. . . Banting died as he had lived —in the service of his country and of humanity. . . The Banting Institute, the Banting Foundation, and the Banting Memorial Lectureship, have been created in the University of Toronto as tangible tributes to him, but his name will live for ever in the hearts of successive generations of diabetics and in the minds of young investigators who will be stimulated by his brilliant and fearless career.

Not long before his death, Fred Banting and I discussed working again together in the laboratory. We had selected a problem in carbohydrate metabolism and had planned a co-ordinated surgical and chemical research.

6. Work since 1945

When Dr Gerald A. Wrenshall joined our Department, we began a study of the initial changes in the blood sugar of the starved anaesthetized dog after alloxan administration (Wrenshall, Collins-Williams & Best, 1950). Dr Wrenshall's findings that there is little or no insulin in the pancreas of "growth onset" cases and that approximately 40% of the normal amount remains in "adult onset" diabetics have stimulated a great deal of productive work (Wrenshall, Bogoch & Ritchie, 1952).

By 1945 I had resumed an active interest in insulin and gave the Banting Memorial Lecture, "Insulin and diabetes—in retrospect and in prospect", at the University of Toronto (Best, 1945).

Dr Stanley Hartroft joined us after the war. While most of our joint activities were devoted to the choline field, Dr Hartroft did make contributions to the histology of the pancreas. A study of the prevalence of diabetes in an Ontario community (Kenny, Chute & Best, 1951) revealed 54 diabetics in a population of 4,419 persons tested, an incidence of 1.2%. Twenty-one of these cases had previously been undiagnosed.

In my Banting Memorial Lecture before the American Diabetes Association (Best, 1952), the early work was

reviewed and some findings on the growth effect of insulin were presented. My colleagues, J. M. Salter, J. Campbell, and C. K. Gorman have found that, in both the hypophysectomized, alloxan-diabetic rat and hypophysectomized-depancreatized dog on a constant dietary intake, when small amounts of insulin are given alone, more nitrogen is retained than when small amounts of somatotropin are given alone. In the presence of small amounts of insulin, somatotropin may have a much greater effect than further amounts of insulin. Similar findings had been reported previously by Lukens and his group in hypophysectomized-depancreatized cats. Both somatotropin and insulin obviously need a source of fuel for the demonstration of their growth effects. Carbohydrate is an important fuel on which insulin relies, whether obtained from the diet or by gluconeogenesis. Depot fat, when it is present, is made available when somatotropin is given, but when the fat depots are depleted the growth effect of somatotropin disappears unless extra calories are provided in the diet. These findings of Greenbaum (1953) and Greenbaum & McLean (1953) in Young's laboratory have been confirmed and extended by Gorman and Salter (unpublished data) in ours. In studies on hypophysectomized animals with the pancreas intact, the effects of diet alone cannot be separated from the effects of insulin which will be liberated in varying amounts.

During the last forty years a number of scientists who are themselves diabetics have come to work with us and we usually have three or four of these highly motivated individuals on our staff. We have, at present, ten medical graduates among those who are working toward their Ph.D. in physiology. A number of these students are working in the field of insulin and experimental diabetes.

It has been my extraordinary good fortune to know personally almost all the scientists who have made significant contributions to our knowledge of insulin during these forty years. I have visited many of them in their own laboratories and a number have lectured or worked with us in Toronto. These two score years have seen great progress and have left us with many wonderful new problems.

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CHEMISTRY OF INSULIN*

F. SANGER Ph.D. F.R.S.

Department of Biochemistry
University of Cambridge

1 Dinitrophenyl method
2 Separating the peptide chains
3 Results of acid hydrolysis
4 Sequences of fraction *B*
5 Sequence of fraction *A*
6 Arrangement of disulphide bridges
7 Biological activity and chemical structure
References

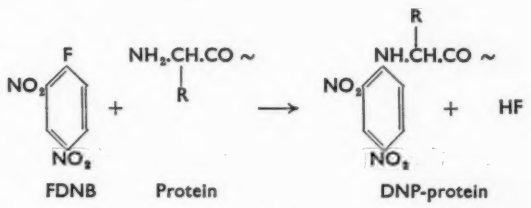
In 1943 the basic principles of protein chemistry were firmly established. It was known that all proteins are built up from amino-acid residues bound together by peptide bonds to form long polypeptide chains. Twenty different amino acids are found in most mammalian proteins, and by analytical procedures it was possible to say with reasonable accuracy how many residues of each one were present in a given protein. Practically nothing, however, was known about the relative order in which these residues were arranged in the molecules. This order seemed to be of particular importance since proteins, although they all contain approximately the same amino acids, differ markedly in both physical and biological properties. It was thus concluded that these differences were dependent on the different arrangement of the amino-acid residues in the molecules. Although very little was known about amino-acid sequence, there was much speculation in this field. The most widely discussed theory was that of Bergman & Niemann (1936), who suggested that the amino acids were arranged in a periodic fashion, the residues of one type of amino acid occurring at regular intervals along the chain. At the other extreme there were those who suggested that a pure protein was not a chemical individual in the classical sense but consisted of a random mixture of similar individuals.

Owing largely to the work of Chibnall and his colleagues (Chibnall, 1942), insulin had been studied in considerable detail. It has a somewhat simpler composition than most proteins in that two of the commonly occurring amino acids, tryptophan and methionine, are absent, and an accurate analysis was available. Moreover, using the Van Slyke procedure, Chibnall had shown that insulin was peculiar in having a high content of free α-amino groups. This indicated that it was composed of relatively short polypeptide chains, since free α-amino groups would be found only on those residues (the *N*-terminal residues) which were present at one end of a chain. Thus the number of chains could be determined from the number of these *N*-terminal residues. The nature of one of these *N*-terminal residues was in fact known. Jensen & Evans (1935) had shown that the phenylhydantoin of phenylalanine could be isolated from an acid hydrolysate of insulin that had been treated with phenyl*iso*cyanate, thus indicating that phenylalanine was at the end of one of the chains. At that time this was the only case where the position of an amino acid in a protein was known.

There was considerable doubt about the actual molecular weight of insulin and hence about the number of amino-acid residues present. Values varying from 36,000 to 48,000 derived by physical methods had been reported, but it was shown by Gutfreund (1948) that these high values resulted from the aggregation of molecules of weight about 12,000, which were conveniently termed "sub-units". If this were so, then there were about 100 amino-acid residues in the molecule or sub-unit. More recently, Harfenist & Craig (1952) have shown that the actual value of the molecular weight is about 6,000, so that the molecule must contain about 50 amino-acid residues; however, during most of our work the molecular weight was believed to be 12,000.

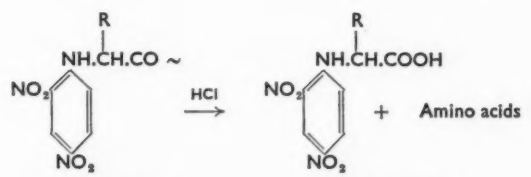
1. Dinitrophenyl Method

In order to study in more detail the free amino groups of insulin and other proteins, a general method for labelling them was worked out (Sanger, 1945). This was the dinitrophenyl (DNP) method. The reagent used was 1:2:4-fluorodinitrobenzene (FDNB), which reacts with the free amino groups of a protein or peptide to form a DNP-derivative (I).



(I)

The reaction takes place under mildly alkaline conditions which normally do not cause any breakage of the peptide bonds. The DNP-protein is then subjected to hydrolysis with acid which splits the peptide bonds in the chain, leaving the *N*-terminal residue in the form of its DNP-derivative (II).



(II)

The DNP-amino acids are bright yellow substances and can be separated from the unsubstituted amino acids by extraction with ether. They could be fractionated by partition chromatography, a method which had just been introduced by Gordon, Martin & Synge (1943) at that time. The DNP-amino acids could then be identified by comparison of their

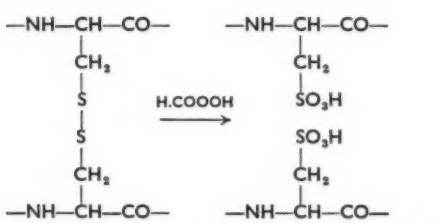
* Largely based on the lecture given before the Swedish Academy in Stockholm on the 12 December 1958, when Dr Sanger was awarded the Nobel Prize for Chemistry in recognition of his elucidation of the chemical structure of the insulin molecule. (By permission of the Editor of *Les Prix Nobel.*)—Ed.

chromatographic rates with those of synthetic DNP-derivatives. In the original work on insulin, silica-gel chromatography was used, though more recently other systems, particularly paper chromatography, have been found more satisfactory. When the DNP-derivatives had been separated and identified they could be estimated colorimetrically.

When the method was applied to insulin, three yellow DNP-derivatives were found in the hydrolysate of the DNP-insulin. One of these was not extracted into ether; this was ε-DNP-lysine, which was formed by reaction of the FDNB with the free ε-amino group of lysine residues which are bound normally within the polypeptide chain. The others were identified as DNP-phenylalanine and DNP-glycine, and estimation on the basis of an assumed molecular weight of 12,000 showed that there were two residues of each. This suggested to us that insulin was composed of four polypeptide chains, two with phenylalanine and two with glycine end-groups. This method has now been applied widely to many proteins and peptides and, together with the Edman phenyl*iso*thiocyanate method (Edman, 1950), is the standard method for studying *N*-terminal residues. In general it has been found that the chains of other proteins are much longer than those of insulin. All pure proteins appear to have only one or two *N*-terminal residues.

2. Separating the Peptide Chains

It seemed probable that the chains of insulin were joined together by the disulphide bridges of cystine residues. Insulin is relatively rich in cystine, and this was the only type of cross-linkage that was definitely known to occur in proteins. It was thus next attempted to separate the peptide chains by splitting the disulphide bridges. Earlier attempts to do this by reduction to —SH derivatives had not proved successful and had given rise to insoluble products which were probably the result of some type of polymerization. More satisfactory results were obtained by oxidation with performic acid (Sanger, 1949a). The cystine residues were converted to cysteic acid residues, and thus the cross-links were broken (III). Performic acid also reacts with residues of methionine and tryptophan, the two amino acids which fortunately were absent from insulin.



(III)

From the oxidized insulin two fractions could be separated by precipitation methods. One (fraction *A*) contained glycine; the other (fraction *B*), phenylalanine *N*-terminal residues. Fraction *A* was acidic and had a simpler composition than insulin in that the six amino acids—lysine, arginine, histidine, phenylalanine, threonine, and proline—were absent from it. It thus had no basic amino acids; these were found only in fraction *B*. From a quantitative determination of the end-groups it was concluded that fraction *A* contained about 20 residues per chain, four of these being cysteic acid, and that fraction *B* had 30 residues, two of which were cysteic acid. Since the yield of each fraction was greater than 50% in terms of the *N*-terminal residues present and since the fractions appeared to be homogeneous, it seemed likely that there was only one type of glycyl chain and one type of phenylalanyl chain. This was confirmed by a study of the *N*-terminal sequences (Sanger, 1949b).

3. Results of Acid Hydrolysis

When the DNP-derivative of fraction *B* was subjected to complete acid hydrolysis, DNP-phenylalanine was produced. If, however, it was subjected to a milder acid treatment so that only a fraction of the peptide bonds was split, DNP-phenylalanine peptides were produced which contained the amino-acid residues near to the *N*-terminal end, and by an analysis of these peptides it was possible to determine the *N*-terminal sequence to four or five residues along the chain. The results for fraction *B* are shown in Table I. It was concluded from these results that all the *N*-terminal phenylalanine residues of insulin were present in the sequence Phe.Val.Asp.Glu. This suggested that if there were in fact two phenylalanyl chains, then these two were identical. Similar results were obtained with fraction *A*, and it was shown that the *N*-terminal glycine residues were present in the sequence Gly.Ileu.Val.Glu.Glu.

These results, besides giving information about the position of certain residues in the polypeptide chains, showed for the first time that the molecule was composed of only two types of chains and that if the molecular weight were 12,000, as was

TABLE I. Results of Partial Acid Hydrolysis of the DNP-Derivative of Fraction *B*

(The yield from DNP-insulin is expressed in moles of peptide as a percentage of the total *N*-terminal phenylalanine residues of insulin.)

Peptide	Products of complete hydrolysis of peptide	Products of partial hydrolysis	Structure	Yield from DNP-insulin
B1	DNP-phenylalanine		DNP—Phe	13
B2	DNP-phenylalanine, valine	B1	DNP—Phe.Val	16
B3	DNP-phenylalanine, valine, aspartic acid	B1, B2	DNP—Phe.Val.Asp	13
B4	DNP-phenylalanine, valine, aspartic acid, glutamic acid	B1, B2, B3	DNP—Phe.Val.Asp.Glu	30
Other bands giving B4 on partial hydrolysis				20
Total				92

then believed, then the molecule was built up of two identical halves. The alternative conclusion was that the actual molecular weight was 6,000, and this was later shown to be the case. At any rate the structural problem was somewhat simplified, since we were now concerned with determining the sequence in two chains containing 20 and 30 residues, respectively.

The main technical problem was the fractionation of the extremely complex mixtures that resulted from partial hydrolysis of a protein. However, Consden, Gordon, Martin & Synge (1947) had shown that small peptides could be well fractionated by paper chromatography and had determined the sequence in the pentapeptide gramicidin S from the composition of peptides produced on acid hydrolysis.

At this point (1949) I was joined by Hans Tuppy, who came to work in Cambridge for a year. Although we did not seriously envisage the possibility of being able to determine the whole sequence of one of the chains within a year, we considered it worth while to investigate the small peptides from an acid hydrolysate, using essentially the methods that had been applied to gramicidin S. Studies were initiated on both the chains at the same time, but it soon became clear that there would be more difficulties with fraction *A*, although it was the shorter chain, and the work on fraction *B* progressed so favourably and Tuppy worked so hard that by the end of the year we were virtually able to deduce the whole of the sequence of its 30 residues (Sanger & Tuppy, 1951a, 1951b).

4. Sequences of Fraction *B*

Fraction *B* was subjected to partial hydrolysis with acid. Since the mixture was too complex for direct analysis by paper chromatography it was necessary to carry out certain preliminary group separations in order to obtain fractions containing 5–20 peptides that could then be separated on paper. This was accomplished by ionophoresis, ion-exchange chromatography, and adsorption on charcoal. These simplified mixtures were then fractionated by two-dimensional paper chromatography. The peptide spots were cut out, and the material was eluted from the paper, subjected to complete hydrolysis, and analysed for its constituent amino acids. Another sample of the peptide was then investigated by means of the DNP technique to determine the *N*-terminal residue. Table II illustrates the results obtained with a very acidic fraction obtained by ion-exchange chromatography. This contained only peptides of cysteic acid. Since there are only two such residues in fraction *B*, all these peptides must fit into two sequences. The way in which the two sequences Leu.$CySO_3H$.Gly and Leu.Val.$CySO_3H$.Gly were deduced from the results obtained with the peptides is illustrated in Table II.

TABLE II. Cysteic Acid Peptides Identified in a Partial Acid Hydrolysate of Fraction *B*

(The inclusion of residues in parentheses indicates that their relative order is not known.)

Peptides	
$CySO_3H$.Gly	$CySO_3H$.Gly
Val.$CySO_3H$	Leu.$CySO_3H$
Val.($CySO_3H$,Gly)	Leu.($CySO_3H$,Gly)
Leu.(Val,$CySO_3H$)	
Leu.(Val,$CySO_3H$,Gly)	
Sequences deduced	
Leu.Val.$CySO_3H$.Gly	Leu.$CySO_3H$.Gly

In this way about 45 peptides were identified in various fractions of the partial acid hydrolysate, and the following five sequences were deduced as being present in the phenylalanyl chain:

Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly (*N*-terminal sequence)	(1)
Gly.Glu.Arg.Gly	(2)
Thr.Pro.Lys.Ala	(3)
Tyr.Leu.Val.$CySO_3H$.Gly	(4)
Ser.His.Leu.Val.Glu.Ala	(5)

These five sequences contain all but four of the amino-acid residues of fraction *B*. It was not possible to determine from the small peptides derived from acid hydrolysates the position of the remaining four residues or the manner in which the five sequences given above were joined together. There were two reasons for this. First, there was considerable technical difficulty involved in fractionating the peptides containing two or more of the non-polar residues such as tyrosine or leucine. It happened that these residues were grouped together in the phenylalanyl chain (see below) and gave rise to a mixture of peptides that moved fast on paper chromatograms and were not well resolved. The second difficulty was due to the great lability in the presence of acid of the bonds involving the amino groups of the serine and threonine residue. It was never possible to find a peptide containing this bond and hence to know what residues preceded the serine and threonine.

It was thus necessary to use another hydrolytic agent that would differ in specificity from concentrated acid. Hydrolysates prepared by the action of dilute acid at high temperatures or of alkali were studied but yielded little further information. Much more successful, however, was the use of proteolytic enzymes (Sanger & Tuppy, 1951b). Initially we had refrained from using them, since it was considered that they might bring about re-arrangement of the peptide bonds by transpeptidation or by actual reversal of hydrolysis. Subsequent work has, however, shown that this is not a very serious danger and that in fact proteolytic enzymes are the most useful hydrolytic agent for studies of amino-acid sequences.

Proteolytic enzymes are much more specific than is acid, since only a few of the peptide bonds are susceptible. They give rise to larger peptides, which in general are more difficult to fractionate by paper chromatography. However, there are relatively few of them, so the mixtures are less complex. In this initial work we used essentially the same methods for studying the enzymic peptides that we had used for the acid ones, depending largely on paper chromatography for fractionation, although more recently it has been shown that better separations can be obtained by ion-exchange chromatography and by ionophoresis.

As an example we may consider a peptide *B*p3 obtained by the action of pepsin. It had the following composition: Phe.($CySO_3H$,Asp,Glu,Ser,Gly,Val,Leu,His). Of these components the most important are aspartic acid and serine, since they occur only once in the chain. Aspartic acid is present only in the *N*-terminal sequence (1), and serine is in sequence (5). This shows that all of sequence (1) and at least the *N*-terminal part of sequence (5) are present in peptide *B*p3. That none of the other sequences are present follows from the fact that *B*p3 contains no arginine (sequence (2)); threonine, proline, or lysine (sequence (3)); or tyrosine (sequence (4)). One may thus conclude that the two sequences are joined together. By studying other peptides obtained by the action

of pepsin, trypsin, and chymotrypsin, it was possible to find out how the various sequences were arranged and to deduce the complete sequence to the phenylalanyl chain, which is as follows: Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly.Ser.His.-Leu.Val.Glu.Ala.Leu.Tyr.Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.-Phe.Phe.Tyr.Thr.Pro.Lys.Ala.

In this work many more peptides were studied from both acid and enzymic hydrolysates than were actually necessary to deduce the sequence. This was considered essential since the methods used were new and were qualitative rather than quantitative. The fact that all the peptides fitted into the unique sequence given above added further proof of its validity.

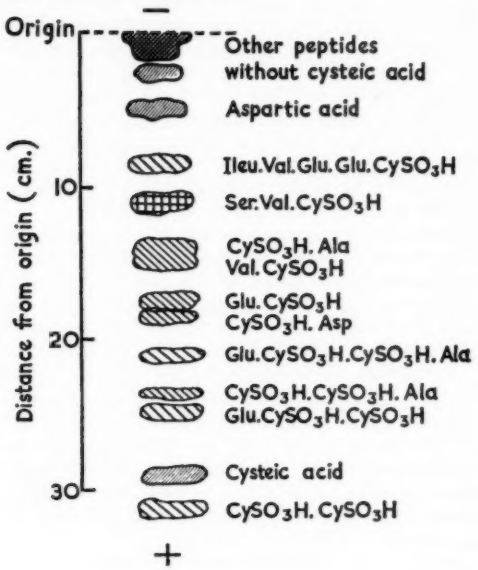
5. Sequence of Fraction *A*

Essentially similar methods were used to determine the sequence of fraction *A* (Sanger & Thompson, 1953). Although it was the shorter of the two chains, the determination of its structure was more difficult. Fraction *B* contains several residues that occur only once in the molecule, and this helps considerably in interpreting the results, whereas fraction *A* has only a few such residues, and these are all near one end. Also, fraction *A* is much less susceptible to enzymic hydrolysis. It is not attacked by trypsin, and there is a sequence of 13 residues which is not split either by chymotrypsin or by pepsin. Considerable difficulty was at first experienced with the cysteic acid peptides. Fraction *A* contains the sequence $CySO_3H$.-$CySO_3H$ and this gave rise to a number of peptides which were very soluble in water and which would not fractionate easily by paper chromatography. However, it was found that by paper ionophoresis at pH 2.75, they could be well separated, since they were the only acidic peptides present. At this pH, —COOH groups are uncharged, while —SO_3H groups carry a negative, and —NH_2 groups a positive, charge. Peptides without cysteic acid were all positively charged, those with one cysteic acid were neutral and could be separated as a group, and those with two cysteic acids were negatively charged. If a slightly higher pH (3.5) is used for the ionophoresis, the —COOH groups become slightly charged and all the peptides containing one cysteic acid residue move slowly towards the anode and can be fractionated in this way. This method was found very useful for the separation and identification of cysteic acid peptides. Figure 1 is a tracing of an ionogram of an acid hydrolysate of fraction *A* achieved in this way.

The sequence of fraction *A* was found to be as follows: Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$.-Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.$CySO_3H$.Asp.

When a protein is hydrolysed with strong acid, it gives rise not only to amino acids but also to a certain amount of ammonia. This is present in the form of amide groups on some of the aspartic and glutamic acid residues. It was thus necessary to determine the position of these groups (Sanger, Thompson & Kitai, 1955). This was done by studying the ionophoretic rates and amide contents of peptides derived from enzymic hydrolysates, since the amide groups are not split off by enzymes, whereas they are by acid. The position of the amide groups is indicated in fig. 2 by the symbol NH_2.

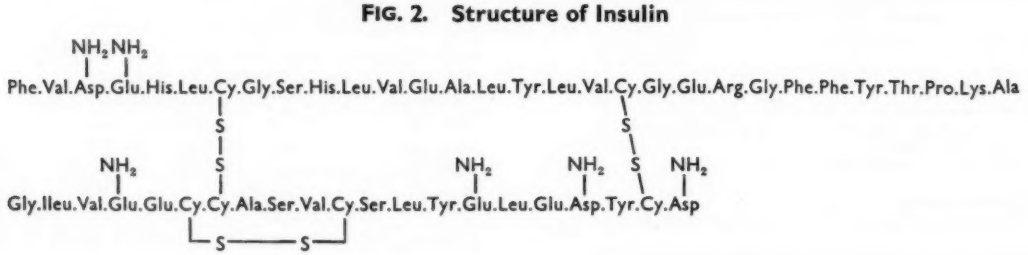
FIG. 1. Ionophoresis of Partial Acid Hydrolysate of Fraction *A* at pH 3.5, Showing Separation of Cysteic Acid Peptides



6. Arrangement of Disulphide Bridges

When the structures of the two chains of insulin had been determined, the only remaining problem was to find how the disulphide bridges were arranged. About this time it was shown by Harfenist & Craig (1952) that the molecular weight of insulin was of the order of 6,000 and thus that the molecule consisted of two chains containing three disulphide bridges, and not of four chains as we had originally thought. The fact that fraction *A* contained four cysteic acid residues whereas fraction *B* had only two indicated that two bridges must connect the two chains together and that one must form an

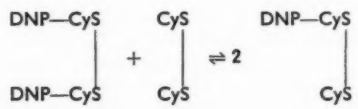
FIG. 2. Structure of Insulin



intra-chain bridge connecting one part of the *A* chain with another part of the same chain.

In order to determine the distribution of the disulphide bridges, it was necessary to isolate, from unoxidized insulin, peptides containing intact cystine residues. These could then be oxidized to give cysteic acid peptides, which could be recognized since they had been found in the hydrolysates of the oxidized chains. However, an unexpected difficulty arose in that, during hydrolysis, a reaction occurred which caused a random re-arrangement of the disulphide bonds, so that cystine peptides were isolated which were not actual fragments of the original insulin, and it would have appeared from the results that every half-cystine was combined to every other half-cystine residue.

This disulphide interchange reaction could be demonstrated and studied when we used as a model system a mixture of cystine and bisDNP-cystine, which reacted together to give monoDNP-cystine:



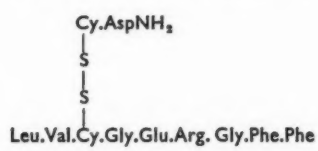
An ether-soluble coloured substance was thus converted to a water-soluble coloured substance, and the course of the reaction could be studied by measuring the distribution of colour between ether and water.

It was found that there were two types of disulphide interchange reactions (Ryle & Sanger, 1955). One took place in neutral and alkaline solution and was catalysed by —SH compounds. It is probably due to initial hydrolysis of the disulphide, which then catalyses a chain reaction:

$$R_1SSR_2 \xrightarrow{OH^-} R_1S^- + R_2SOH$$

$$R_1S^- + R_3SSR_4 \longrightarrow R_1SSR_3 + R_4S^- \ldots$$

In neutral conditions the reaction could be inhibited by —SH inhibitors; hence, it was possible to use enzymic hydrolysis to obtain cystine peptides (Ryle, Sanger, Smith & Kitai, 1955). Thus, for instance, with chymotrypsin, a peptide was obtained which on oxidation gave the two cysteic acid peptides $CySO_3H$.-Asp.NH_2 and Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe.Phe. The structure of the cystine peptide was thus:



establishing the presence of a disulphide bridge between the two half-cystine residues nearest the *C*-terminal ends of the two chains.

It was not, however, possible to determine the positions of the other two disulphide bonds by means of enzymic hydrolysis, since no enzyme would split between the two consecutive half-cystine residues of the *A* chain. It was therefore necessary to re-investigate the possibility of using acid hydrolysis.

The disulphide interchange reaction that occurred in acid solution was found to be different from that occurring in neutral and alkaline solution and instead of being catalysed by —SH compounds was actually inhibited by them. This not only showed that a different reaction was involved but it also made it possible to prevent its occurring during acid hydrolysis. Thus, when insulin was treated with concentrated acid to which a small amount of thioglycolic acid was added, cystine peptides could be isolated which were in fact true breakdown products and from which the distribution of the remaining two disulphide bonds could be deduced. These are shown in fig. 2, which shows the complete structure of insulin.

Of the various theories concerned with protein chemistry, our results supported only the classical peptide hypothesis of Hofmeister and Fischer (see Fischer, 1906). The fact that all our results could be explained on this theory added further proof, if any were necessary, of its validity. The results also showed that proteins are definite chemical substances possessing a unique structure in which each position in the chain is occupied by one, and only one, amino-acid residue.

Examination of the sequences of the two chains reveals no evidence of periodicity of any kind, nor does there seem to be any basic principle which determines the arrangement of the residues. They seem to be put together in an order that is random, but nevertheless unique and most significant, since on it must depend the important physiological action of the hormone.

7. Biological Activity and Chemical Structure

As yet little is known about the relationship of the physiological action of insulin to its chemical structure. Before much was known about the structure many experiments were done, especially by Freudenberg and his collaborators (Freudenberg & Wegman, 1935), in which insulin was treated with various reagents that were specific for one type of group and the changes in biological activity were observed. It has long been realized that the disulphide bridges are important and must remain intact for activity. When they are broken by reduction (Stern & White, 1937) or oxidation (Freudenberg & Wegman, 1935) there is immediate inactivation. Of particular interest is the intra-chain disulphide bridge that forms a ring in the *A* chain. The ring is the same size as similar disulphide rings present in two other hormones, oxytocin and vasopressin from the posterior pituitary, and it is tempting to speculate whether there may be some biological significance in this structure that is found in the three hormones. There is, however, no experimental evidence that this disulphide bond is particularly important. The amino acids forming the ring in oxytocin and vasopressin are quite different from those in insulin.

Acetylation, which blocks only the free amino groups, causes no change in activity (Stern & White, 1938), thus suggesting that free amino groups are not essential constituents of any part of the molecule whose integrity is necessary for biological activity to be manifest. On the other hand, esterification, which blocks the free carboxyl groups, caused loss of activity; this could be recovered by removal of the ester groups with alkali (Carr, Culhane, Fuller & Underhill, 1929). Similarly the phenolic hydroxyl groups of the tyrosine residues appear to be important, since iodination leads to loss of activity (Harington & Neuberger, 1936). One drawback of this type of method is that there is no way of knowing which

particular residue is involved. Thus, for instance, there are six carboxyl groups in insulin, and one cannot tell if all or only one are essential for activity.

Now that the structure is known, it has been possible to devise experiments in which known parts of the molecule are broken off by controlled enzymic hydrolysis, and the residue tested for activity.

When insulin is treated with carboxypeptidase, the alanine residue in the *C*-terminal position of the phenylalanyl chain is rapidly split off and there is no alteration of biological activity (Harris & Li, 1952; Nicol, 1960). This residue can also be split off by the controlled action of trypsin with the same result. The alanine is thus of no importance for activity. The other *C*-terminal residue of insulin, asparagine, is much more resistant to carboxypeptidase. Nevertheless, under special conditions it can be removed, and its removal, unlike that of alanine, leads to loss of biological activity (Harris & Li, 1952; Nicol, 1960). It is interesting that this residue is in the sequence Tyr.CyS.Asp.NH_2, which contains a phenolic hydroxyl, a disulphide and a free carboxyl, the three groups that appear to be essential for activity. It is possible that this part of the molecule may be of particular biological importance. However, there is no evidence that it is in fact these particular residues which are involved when insulin is inactivated by iodination, reduction or esterification.

When insulin is treated with trypsin two bonds are split. The Lys—Ala bond at the *C*-terminal end of the phenylalanyl chain is split relatively rapidly, giving rise to the same active fragment that was obtained by the action of carboxypeptidase. Further treatment with trypsin leads to a slow splitting at the Arg—Gly bond in the *B* chain, giving rise to free alanine, a heptapeptide Gly.Phe.Phe.Tyr.Thr.Pro.Lys, and the residue of the molecule (DHA insulin). The heptapeptide was inactive, but the DHA insulin, which could be separated chromatographically from any unchanged insulin, had about 15% of the normal activity (Nicol, 1960). This result would seem to indicate that the part of the *B* chain represented by the above heptapeptide is not an essential part of the active centre, though its presence does affect the activity quantitatively.

When insulin is treated with leucine aminopeptidase, a number of residues are split off from the *N*-terminal end of both chains. By comparing the activity of a digest with the amino acids liberated, Smith, Hill & Borman (1958) concluded that the first six residues of the *B* chain are not essential for activity.

Another approach to the problem of the physiological activity of insulin is to study the insulins from different animal species (Brown, Sanger & Kitai, 1955; Harris, Sanger & Naughton, 1956). Since all insulins show the same activity, it could be concluded that differences would be found only in parts of the molecule that were not important for activity.

All the results given above were obtained with insulin from cattle. When insulins from four other species were studied by essentially the same methods, it was found that the whole of the *B* chain was identical in all species, and the only differences that were found were in the three amino acids contained within the disulphide ring of the *A* chain, which in the cattle are: Ala.Ser.Val, and in the other species are: Thr.Ser.Ileu (pig); Ala.Gly.Val (sheep); Thr.Gly.Ileu (horse); Thr.Ser.Ileu (whale). These results suggest that the exact structure of the residues in this position is not important for biological activity, but it does not necessarily follow that the whole of the rest of the molecule is important. Recently L. F. Smith (private communication, 1960) has studied human and rabbit insulin. Both have the same sequence as the pig (Thr.Ser.Ileu) within the disulphide ring but a difference was found in another part of the molecule, the *C*-terminal residue of the *B* chain, which in the other species is alanine, in the rabbit is serine and in man is threonine. This supports the conclusion from the carboxypeptidase experiments that the *C*-terminal alanine is not essential for biological activity. The structure of the human insulin is of particular medical interest. It will be noticed that it is different from the various commercially available insulins but most closely resembles that from the pig.

The determination of the structure of insulin clearly opens up the way to similar studies on other proteins, and already such studies are going on in a number of laboratories. These studies are aimed at determining the exact chemical structure of the many proteins that go to make up living matter and hence at understanding how these proteins perform their specific functions on which the processes of life depend. One may also hope that studies on proteins may reveal changes that take place in disease, and that our efforts may be of more practical use to humanity.

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THE CHEMISTRY OF PITUITARY POLYPEPTIDE HORMONES

IEUAN HARRIS M.Sc. Ph.D.

Department of Biochemistry
University of Cambridge

1 Oxytocin and vasopressin
  a Chemistry of oxytocin
  b Chemistry of vasopressin
  c Biological properties of oxytocin and vasopressin
  d Relationships between chemical structure and biological activity
  e Species variations
2 Adrenocorticotropic hormone (ACTH, corticotropin)
  a Chemistry of corticotropins
  b Species variations among corticotropins
  c The structure and activity of corticotropins
3 Melanocyte-stimulating hormones
  a Chemistry of melanocyte-stimulating hormones
  b β-Melanocyte-stimulating hormones
  c α-Melanocyte-stimulating hormone
  d Melanocyte-stimulating activity of corticotropin
  e The chemical basis of melanocyte-stimulating activity
  f Species differences among melanocyte-stimulating hormones
References

The pituitary gland fulfils a number of important roles which are essential for the normal life of an organism, and considerable progress has been made during the past decade towards the isolation and chemical characterization of some of the biologically active substances which have been found in extracts of mammalian pituitary glands.

Over 60 years have elapsed since Oliver & Schäfer (1895) and Howell (1898) found that extracts of the posterior pituitary were effective in raising the blood pressure in mammals. Once this original observation had been made, several other biological effects were noted and ascribed to the posterior pituitary: these include the uterine-contracting or oxytocic effect (Dale, 1906), the milk-ejecting effect (Ott & Scott, 1910), the lowering of blood pressure in birds (Paton & Watson, 1912) and antidiuresis (von den Velden, 1913). Extracts of the intermediate lobe were shown to regulate the pigmentation of amphibia (e.g., Atwell, 1919; Hogben & Winton, 1922; Smith & Smith, 1923) and, during the course of a study of anterior pituitary function, Smith (1926, 1927) demonstrated the presence of a substance which controlled both the size and the functioning of the adrenal cortex.

Many other biologically active substances have subsequently been found to occur in the anterior pituitary as well as in other mammalian organs. However the present paper will be limited to a discussion of the peptide hormones, oxytocin, vasopressin, corticotropin and the melanocyte-stimulating hormones, which collectively are responsible for all the biological effects noted in the preceding paragraph.

Many attempts were made during the first half of the century to isolate the various active principles of the pituitary in pure form. These efforts met with only limited success and in retrospect it is clear that their successful outcome had to await the development and application of new and more powerful methods of fractionation, such as zone electrophoresis (see Tiselius & Flodin, 1953), ion-exchange chromatography (see Moore & Stein, 1956) and counter-current distribution (Craig, 1944). Once these methods became available they were quickly applied to the fractionation of polypeptide hormones from pituitary extracts, and the first major success was achieved when pure oxytocin and vasopressin were isolated from ox pituitary glands by du Vigneaud and his colleagues (Livermore & du Vigneaud, 1949; Turner, Pierce & du Vigneaud, 1951). During the course of the next few years pure corticotropins (White & Fierce, 1953; Bell, 1954; Li, Geschwind, Levy, Harris, Dixon, Pon & Porath, 1954; Dixon & Stack-Dunne, 1955) and melanocyte-stimulating hormones (Benfey & Purvis, 1955; Lerner & Lee, 1955; Lee & Lerner, 1956; Porath, Roos, Landgrebe & Mitchell, 1955; Geschwind, Li & Barnafi, 1956, 1957a) were also isolated.

Thus after several decades of frustration some of the biological activities in pituitary extracts were given a chemical identity and, for the first time, it became possible to investigate their chemical structures and to carry out meaningful biological studies on hormones of established purity. In order to do so, however, thousands of glands had to be collected and processed and it was clear that chemical studies would have to be undertaken on milligram quantities of the polypeptide substances.

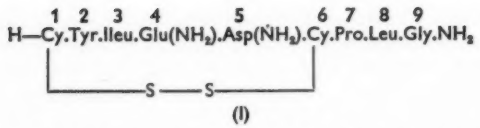
Fortunately, micro-chemical techniques for the study of peptide structure were already available. These included partition chromatography on paper (Consden, Gordon & Martin, 1944) and column chromatography (Moore & Stein, 1949, 1951) for the separation, identification and estimation of amino acids; and methods of end-group analysis (Sanger, 1945; Edman, 1950), and of partial hydrolysis of polypeptide chains (see Sanger, 1952). The successful application of these techniques by Sanger and his colleagues in their pioneer studies on the pancreatic hormone, insulin[1], greatly facilitated the work which ultimately led to the elucidation of the chemical structures of oxytocin, vasopressin, corticotropin and the melanocyte-stimulating hormones.

1. Oxytocin and Vasopressin

a. Chemistry of Oxytocin

The pure hormone isolated from ox pituitaries was found to be a polypeptide derivative consisting of eight amino acids, leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and cystine, in equimolar proportions; in addition, three molar equivalents of ammonia were also produced when it was subjected to hydrolysis with acid (Pierce & du Vigneaud, 1950; Privat de Garilhe, Maier-Hüser & Fromageot, 1951). The structure of the peptide hormone was derived from the results of studies of partial hydrolysis carried out on the native hormone as well as on the derivative formed by oxidation of its disulphide bond with performic acid. In this way it was shown to be an octapeptide amide consisting of a pentapeptide ring structure linked to a tripeptide amide side-chain as shown in formula I (Tuppy, 1953; du Vigneaud, Ressler & Trippett, 1953). The validity of the structure which had been deduced from the results of degradative experiments was confirmed in an elegant way

[1] See Sanger, p. 183 of this number of the Bulletin.—Ed.

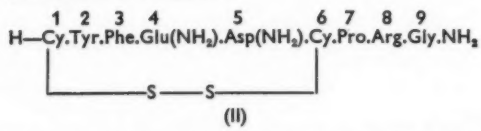


(I)

when a synthetic product based on the above structure was found to be indistinguishable from the natural hormone both in its physico-chemical and biological properties (du Vigneaud, Ressler, Swan, Roberts, Katsoyannis & Gordon, 1953).

b. Chemistry of Vasopressin

When methods similar to those which had proved successful in the case of oxytocin were applied to ox vasopressin, this was shown to be a cyclic octadecapeptide amide of the same general structure as oxytocin (Acher & Chauvet, 1953; du Vigneaud, Lawler & Popenoe, 1953). Six amino acids, cystine, glycine, glutamine, asparagine, proline and tyrosine, were common to both hormones, but vasopressin differs from oxytocin in containing phenylalanine, instead of isoleucine (position 3) in the cyclopentapeptide ring, and arginine instead of leucine (position 8) in the tripeptide amide side-chain, as shown in formula II.



(II)

When vasopressin was isolated from pig glands it was found to contain lysine instead of arginine in position 8 in the molecule (du Vigneaud, Lawler & Popenoe, 1953). Subsequently both lysine-vasopressin and arginine-vasopressin were synthesized (du Vigneaud, Gish & Katsoyannis, 1954; Bartlett, Jöhl, Roeske, Stedman, Stewart, Ward & du Vigneaud, 1956) and the synthetic products were again found to be indistinguishable both chemically and biologically from the naturally occurring pig hormone and ox hormone respectively.

c. Biological Properties of Oxytocin and Vasopressin

A study of the biological properties of both natural and synthetic oxytocins and vasopressins settled many arguments concerning the number of different biologically active molecules which are present in extracts of the posterior lobe. Thus, in addition to its oxytocic or uterine-contracting activity, pure oxytocin was also found to be the factor mainly responsible for lowering the blood pressure in birds (avian depressor effect) and for milk-ejecting activity in rabbits. Pure oxytocin was found also to possess 1–2% of the pressor and antidiuretic activities of pure vasopressin. This degree of activity, although small, was significant. These two activities are normally associated with vasopressin, and at one time it was thought that oxytocin as isolated from the gland might have been contaminated with a trace of vasopressin; however, when synthetic oxytocin became available it was established beyond doubt that these activities are intrinsic properties of the oxytocin structure (du Vigneaud, 1954).

Similarly, synthetic vasopressin, the main pressor and antidiuretic hormone of the pituitary, was found to possess significant amounts of uterine-contracting, milk-ejecting and avian vasopressor activities. It should also be noted that arginine-vasopressin and lysine-vasopressin differ markedly in their relative pressor : antidiuretic potencies (van Dyke, Engel & Adamsons, 1956); in arginine-vasopressin the ratio of the two activities is 1 : 1, whereas in lysine-vasopressin it was found to be 6 : 1.

Biological studies on the two pure hormones have thus shown that collectively they account for all the known biological activities which have been attributed to extracts of the posterior lobe. (See du Vigneaud, 1954.)

d. Relationships between Chemical Structure and Biological Activity

Consideration of the chemical and biological properties of oxytocin and vasopressin reveals a high degree of chemical specificity. Thus, although the two hormones possess very similar chemical structures, the replacement of isoleucine and leucine in the oxytocin molecule by phenylalanine and arginine (or lysine) in vasopressin leads to profound changes in the biological properties of the two molecules. Nevertheless the fact that oxytocin possesses some vasopressin activity, and vice versa, shows that chemical specificity is not an absolute all-or-none phenomenon.

In an endeavour to investigate the effects of specific changes in chemical structure on biological properties, a variety of synthetic analogues of the two hormones have been synthesized. Three such analogues, oxypressin (Katsoyannis, 1957), arginine-vasotocin (Katsoyannis & du Vigneaud, 1958), and lysine-vasotocin (Boissonnas & Huguenin, 1960) are of considerable interest since they represent hybrid molecules, consisting of the vasopressin ring coupled with the oxytocin side-chain, and the oxytocin ring coupled with the vasopressin side-chains, respectively. When the biological properties of these analogues were investigated, it was found that they possessed the various activities of oxytocin and vasopressin in quite different ratios from those found in the two natural hormones. Recently, a substance which appears to be arginine-vasotocin has been found in extracts of fish, frog, and chicken pituitaries (Pickering & Heller, 1959; Sawyer, Munsick & van Dyke, 1959; Chauvet, Lenci & Acher, 1960).

The high pressor activity of arginine-vasotocin relative to that of oxypressin demonstrates the importance of a basic side-chain for pressor activity. For oxytocic activity the integrity of the cyclopentapeptide structure appears to be more important than that of the tripeptide amide side-chain, since arginine-vasotocin retains an appreciable amount of uterine-contracting activity despite the presence of a basic amino acid in its side-chain. The 2-phenylalanine analogue of oxytocin, on the other hand, in which tyrosine (position 2 in the ring) is replaced by phenylalanine, possesses only 5% of the uterine-contracting activity of oxytocin itself, showing that the phenolic hydroxyl group of tyrosine contributes strongly to the activity of the hormone (Bodanszky & du Vigneaud, 1959; Jaquenoud & Boissonnas, 1959). An even more striking demonstration of chemical specificity is the finding that analogues formed by replacing glutamine by *iso*glutamine, and asparagine by *iso*asparagine (Ressler & du Vigneaud, 1957; Lutz, Ressler, Nettleton & du Vigneaud, 1959), and by glutamine (Boissonnas, Guttmann, Jaquenoud & Waller, 1956), are completely inactive.

Boissonnas, Guttmann, Jaquenoud & Waller (1956) have also synthesized a series of analogues in which the isoleucine residue (position 3 in the oxytocin ring) is replaced by leucine, phenylalanine and valine respectively. When assayed on the isolated rat uterus, all three analogues were found to be

considerably less active than oxytocin itself. The valine analogue, however, gave a much higher activity when assayed both on the cat and rat uterus in vivo and was also highly active when assayed for milk-ejection pressure in the rabbit. Guttmann, Jaquenoud & Boissonnas (1957) have also shown that an analogue of oxytocin containing an additional tyrosine residue in its ring is not only inactive but is also a potent inhibitor of oxytocic activity.

e. Species Variations

Oxytocins and vasopressins isolated from ox, pig, sheep (Acher, Chauvet & Lenci, 1959a), horse (Acher, Chauvet & Lenci, 1959b), chicken (Chauvet *et al.* 1960), and human (Light & du Vigneaud, 1958) pituitaries have been found to possess identical chemical structures, with the single exception of pig vasopressin which contains lysine instead of arginine. Clearly the two molecules possess highly specific chemical structures which have been preserved by natural selection, and mutations which may occur in the genes that control their synthesis are unlikely to survive, since they would presumably result in the formation of molecules of altered biological specificity.

2. Adrenocorticotropic Hormone (ACTH, Corticotropin)

The name corticotropin (ACTH) refers to the active principle in the anterior pituitary which stimulates the adrenal cortex. In addition to stimulating the secretion of corticosteroids, corticotropin also depletes the level of adrenal ascorbic acid in hypophysectomized rats, and this effect forms the basis of a sensitive and convenient assay method for the hormone (Sayers, Sayers & Woodbury, 1948).

After the corticotropic activity of anterior pituitary tissue had been discovered (Smith, 1926), numerous unsuccessful attempts were made to isolate the active principle in pure form. Some progress was made when protein preparations possessing corticotropic activity were isolated from both pig and sheep pituitary extracts by Li, Simpson & Evans (1942) and Sayers, White & Long (1943) respectively. Subsequently Li (1947) observed that these "ACTH protein" preparations could be digested with pepsin without loss of activity; and this observation, coupled with the discovery that corticotropic preparations were effective in the clinical treatment of rheumatoid arthritis, gave impetus to renewed efforts to isolate the pure hormone.

A major advance was made when Astwood and his colleagues (Astwood, Raben, Payne & Grady, 1951) showed that the active substance could be separated from ACTH protein, without prior hydrolysis with pepsin, by adsorption on to oxycellulose. Oxycellulose concentrates prepared in this manner were subsequently used as starting materials from which pure corticotropins were isolated from pig (White & Fierce, 1953; Bell, 1954; Dixon & Stack-Dunne, 1955) and from sheep glands (Li *et al.* 1954). More recently pure corticotropins have also been isolated from ox (Li & Dixon, 1956; White & Peters, 1956) and human (Lee, Lerner & Buettner-Janusch, 1959) glands.

a. Chemistry of Corticotropins

As soon as adequate supplies of pure hormone became available, chemical studies were undertaken in three different laboratories. Corticotropins from pig, sheep and ox pituitaries were all found to be straight-chain polypeptides consisting of 39 amino-acid residues. The amino-acid sequence in the pig hormone was determined in the laboratories of the American Cyanamid Company, Stamford, Connecticut (Bell, 1954; Shepherd, Willson, Howard, Bell, Davies, Davis, Eigner & Shakespeare, 1956) and in the Armour Laboratories, Chicago, Illinois (White & Landmann, 1955), while the sequences in the sheep and ox hormones were elucidated by C. H. Li and his colleagues at the University of California (Li, Geschwind, Cole, Raacke, Harris & Dixon, 1955; Li, Dixon & Chung, 1958).

The methods used in the study of corticotropins were essentially similar to those described by Sanger for insulin. Thus polypeptide fragments derived from the various corticotropins by selective hydrolysis with the proteolytic enzymes—trypsin, chymotrypsin, pepsin and subtilisin—were isolated from the respective enzymic digests, and their amino-acid compositions and *N*-terminal and *C*-terminal amino acids were determined. A number of such fragments were then selected for further study; once certain overlapping or "hinge" sequences had been established, the peptides could be lined up in the correct order and the complete sequence of the 39 amino acids deduced from the sequences of the individual peptides. However, whereas the structure of insulin was deduced entirely from the results of degradative studies involving partial hydrolysis with enzymes and acid, newer methods of stepwise analysis (cf. Fraenkel-Conrat, Harris & Levy, 1955) were also used to determine the structure of the corticotropins.

The complete structure of pig corticotropin (Shepherd *et al.* 1956) is given in formula III. A similar structure for the pig hormone was proposed by White & Landmann (1955), except that there was a disagreement concerning the sequence of the amino acids in positions 25–29 of the molecule. We have recently re-investigated the structure of pig corticotropin (Waller & Harris, unpublished results, 1959), and our results support the validity of the structure originally proposed by Shepherd *et al.* (1956).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
H.Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val.Gly.Lys.Lys.Arg.Arg.Pro.Val.Lys.Val.Tyr.Pro.Asp.Gly.Ala.Glu.Asp.Glu.Leu.Ala.Glu.Ala.Phe.Pro.Leu.Glu.Phe.OH

(III)

b. Species Variations among Corticotropins

Corticotropins isolated from sheep, ox and human pituitaries were found to have identical amino-acid compositions; however, they differed from the pig hormone in that they each contained three residues of serine and one of leucine instead of two residues of each amino acid as in the pig hormone. When the amino-acid sequences of the sheep and ox hormones were determined it was clear that the structural differences occurred in positions 31 and 32 (formula III), where the Leu.Ala sequence in pig corticotropin is changed to Ala.Ser in sheep corticotropin, and to Ser.Ala in ox corticotropin, respectively.

In addition, differences in sequence were also noted in positions 25–29, where the sequence Asp.Gly.Ala.Glu.Asp in the pig hormone becomes re-arranged to Ala.Gly.Glu.Asp.-Asp in sheep corticotropin, and to Asp.Gly.Glu.Ala.Glu in ox corticotropin. The sequence of this part of the molecule in human corticotropin has not as yet been determined. These differences in structure, which involve re-arrangements in

sequence of the same five amino-acid residues, may well represent genuine species variations. On the other hand, the sequences in this region of the molecule have proved to be particularly difficult to determine and should be confirmed, preferably by methods which do not require strong acid conditions, before these apparent species variations can be said to be unequivocally established.

c. The Structure and Activity of Corticotropins

The elucidation of the complete amino-acid sequence of corticotropin represents a notable achievement in structural organic chemistry. Pride of achievement was, however, tinged with disappointment when it was realized that earlier predictions concerning its possible molecular size (for example, Li & Pedersen, 1950) had not been confirmed and that consequently the chemical synthesis of the hormone on a commercial scale was not likely to be a practical proposition. There remained the hope that the complete molecule of 39 amino acids might not be essential for biological activity, and this possibility led to many efforts to seek active fragments of the hormone.

Earlier studies carried out on relatively crude corticotropic concentrates had suggested that the activity of the hormone was not destroyed by pepsin (Li, 1947; Brink, Meisinger & Folkers, 1950), and these observations were confirmed when similar studies were carried out on pure corticotropins. Thus Bell, Howard, Shepherd, Finn & Meisenhelder (1956) showed that the major sites of hydrolysis were confined to the *C*-terminal portion (positions 28–39, formula III) of the molecule and that the peptide fragment consisting of the *N*-terminal portion (positions 1–28) retained all the biological properties of the intact hormone molecule. Subsequent studies involving partial hydrolysis with dilute acid showed further that the *N*-terminal peptide consisting of the first 24 amino acids in the molecule also retained biological activity. This peptide of 24 amino acids is the smallest fully active fragment that has been isolated from the natural hormone. Preliminary synthetic studies have shown, however, that the *N*-terminal, icosapeptide (Boissonnas, Guttmann, Waller & Jaquenoud, 1956), and tridecapeptide (Guttmann, 1960) portions of the molecule also possess some biological activity in an in-vitro assay.

The fact that the *N*-terminal portion of the molecule (positions 1–24, formula III) retains the physiological properties of the hormone is of considerable theoretical interest, since the differences in structure which have been encountered among corticotropins from different species occur exclusively in that part of the molecule—between positions 25 and 39—the integrity of which is not essential for biological activity (formula III).

All attempts to degrade corticotropin from the other (*N*-terminal) end of the molecule have invariably led to loss of biological activity. Thus, prolonged digestion with pepsin (which splits the Glu–His bond, positions 5–6 (Bell *et al.* 1956)), partial hydrolysis with aminopeptidase (which led to the removal of the *N*-terminal dipeptide, Ser.Tyr (White, 1955)), mild oxidation of the hormone with periodate (which oxidizes the $CH_2(OH).CH(NH_2)$ moiety of the *N*-terminal serine (Dixon, 1956)), all lead to extensive inactivation of the hormone. The essential role of the *N*-terminal serine was also revealed when *N*-acetylcorticotropin (a derivative in which the α-NH_2 group of the serine is selectively acetylated) was found to be virtually inactive when assayed for corticotropic activity by the Sayers method (Waller & Dixon, 1960).

In summary, the chemical structure which is responsible for corticotropic activity occurs within the *N*-terminal portion (positions 1–24, formula III) of the molecule. It is significant that this particular sequence of 24 amino acids is identical in all known corticotropins although, as we have seen, several differences in structure have been noted in the *C*-terminal portions (positions 25–39) of corticotropins isolated from different mammalian species.

3. Melanocyte-Stimulating Hormones

The presence of a melanocyte-stimulating substance in the intermediate lobe of the pituitary has been recognized for well over 30 years as a result of the investigations of Atwell (1919) and Hogben & Winton (1922), as well as of others, on the ability of pituitary extracts to cause darkening of the skin in certain amphibia, particularly the frog.

Recent interest in this hormone has stemmed largely from speculations concerning its relationship to corticotropin. For example, the possibility has been considered that melanocyte-stimulating hormone could be at least partly responsible for the darkening of the skin which occurs in Addison's disease. In addition, the increased levels of both melanocyte-stimulating hormone and corticotropin in blood, which are characteristic of Addison's disease and of the Cushing syndrome (Johnsson & Högberg, 1952), led to the suggestion that the same hormone molecule might be responsible for both activities (e.g., Sulman, 1952). However, even before corticotropin and melanocyte-stimulating hormones had been isolated in pure form there was strong evidence to support the view that the main melanocyte-stimulating activity of the pituitary was not associated with corticotropin (Reinhardt, Geschwind, Porath & Li, 1952; Landgrebe & Morris, 1955). Nevertheless, when pure corticotropins were isolated they were invariably found to possess a small but constant residual melanocyte-stimulating activity, and this dual activity is now known to be a reflection of the chemical similarities which exist between the two hormone molecules.

a. Chemistry of Melanocyte-Stimulating Hormones

The isolation, from pig pituitary glands, of a highly purified melanocyte-stimulating polypeptide was first reported by Lerner & Lee (1955). Subsequently, what appeared to be a different melanocyte-stimulating substance was isolated independently in three other laboratories (Benfey & Purvis, 1955; Porath *et al.* 1955; Geschwind *et al.* 1956). This apparent discrepancy was resolved when Lee & Lerner (1956) showed that both forms of the hormone were present in the pig pituitary. The highly basic polypeptide which they isolated originally was given the name α-melanocyte-stimulating hormone (α-MSH), while the slightly acidic polypeptide isolated in the other laboratories was called β-melanocyte-stimulating hormone (β-MSH). In the earlier literature melanocyte-stimulating hormone is frequently referred to as intermedin or melanophore-expanding hormone. Geschwind, Li & Barnafi (1957b) have also reported the isolation of β-MSH from ox pituitaries, while more recently Dixon (1960) has isolated the hormone from human glands.

b. β-Melanocyte-Stimulating Hormones

The chemical structure of pig β-MSH was elucidated by Harris & Roos (1956, 1959). Amino-acid analysis indicated that the hormone was an octadecapeptide of the following

composition: $Arg_1Asp_2Glu_2Gly_2His_1Lys_2Met_1Phe_1Pro_3Ser_1Try_1Tyr_1$. The presence of three basic and three aromatic amino acids suggested that β-MSH could be hydrolysed with trypsin and chymotrypsin to give a series of peptide fragments which would be amenable to sequence analysis. The amino-acid sequence of the peptide hormone was ultimately deduced by applying methods of stepwise analysis (see Harris & Roos, 1959) to the intact molecule as well as to the peptide fragments derived from it by enzymic hydrolysis. The complete sequence is given in fig. 1.

The most striking feature of the structure is that it contains a sequence of seven amino acids, Met.Glu.His.Phe.Arg.Try.Gly, which is also present in the corticotropin molecule, and the presence of this common structure provides a chemical basis for the melanocyte-stimulating activity which is an intrinsic property of corticotropin itself.

The same structure for pig β-MSH was also derived by Geschwind *et al.* (1956, 1957a). These authors (Geschwind *et al.* 1957b) also reported the isolation and structure of ox β-MSH, which like the pig hormone proved to be an octadecapeptide, but contains serine instead of glutamic acid in position 2. Human β-MSH, on the other hand, was found to consist of 22 amino-acid residues, and contains the *N*-terminal tetrapeptide Ala.Glu.Lys.Lys in addition to the octadecapeptide structure which it possesses in common with the pig and ox hormones; and, within this common structure, it contains arginine instead of lysine (Harris, 1959b). These results are summarized in fig. 1.

c. α-Melanocyte-Stimulating Hormone

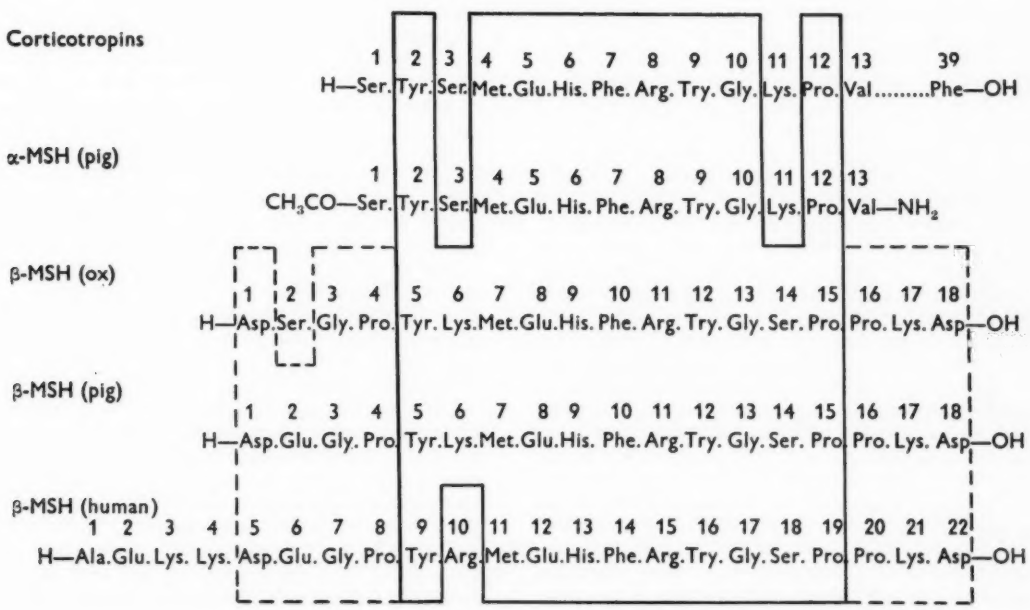
When it was first isolated, α-MSH proved to be so different in physico-chemical properties from the β-MSH's that much speculation ensued concerning its method of isolation and possible significance (cf. Li, 1957). All doubts as to its existence as a separate melanocyte-stimulating hormone in the pig pituitary were, however, dispelled when its chemical composition and structure were established (Harris & Lerner, 1957; Harris, 1959a). α-MSH proved to be a very unusual polypeptide, since it contained neither free *N*-terminal nor *C*-terminal amino acids; structural studies showed that it was an acetyltridecapeptide amide in which the sequence of amino acids was identical with the sequence of the *N*-terminal tridecapeptide part of corticotropin (see fig. 1). This result gave additional emphasis to the striking chemical relationships which exist between the two hormones.

d. Melanocyte-Stimulating Activity of Corticotropin

The amino-acid sequences of all known melanocyte-stimulating hormones are given in fig. 1. The heptapeptide structure, Met.Glu.His.Phe.Arg.Try.Gly, is common to all the molecules and but for the interchange of serine and lysine residues (positions 3 and 11, respectively) in α-MSH and corticotropin, and the replacement of lysine by arginine (position 10) in human β-MSH, the sequence in common would extend to 11 amino acids. Although all these structures possess melanocyte-stimulating activity, corticotropin is considerably less active (*ca* 1% as active as pig β-MSH) than the others in spite of the fact that it contains the entire tridecapeptide structure of the most active molecule, that is, α-MSH.

As a working hypothesis (Harris, 1959a), it was considered that the additional structural features which are essential for corticotropic activity may at the same time inhibit or mask the potential melanocyte-stimulating activity of the corticotropin molecule. The most obvious chemical difference (apart from the disparity in chain-length) is that the terminal serine in corticotropin possesses a free α-NH_2 group, whereas in α-MSH

FIG. 1. Amino-Acid Sequences of Melanocyte-Stimulating Hormones from Mammalian Pituitary Glands


Corticotropins
1 2 3 4 5 6 7 8 9 10 11 12 13 39
H—Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val.....Phe—OH
α-MSH (pig)
1 2 3 4 5 6 7 8 9 10 11 12 13
CH3CO—Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val—NH2
β-MSH (ox)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
H—Asp.Ser.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp—OH
β-MSH (pig)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
H—Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp—OH
β-MSH (human)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
H—Ala.Glu.Lys.Lys.Asp.Glu.Gly.Pro.Tyr.Arg.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp—OH

it occurs as the *N*-acetyl derivative. Could the $CH_3(OH)$. $CH(NH_2)$ structure, which is known to be essential for corticotropic activity, also inhibit the melanocyte-stimulating activity of corticotropin? Or is it the increase in chain-length from 13 to 39 amino acids which makes corticotropin a less potent melanocyte-stimulator than α-MSH?

In an investigation of the relationship between alterations of the serine residue and biological activity, Waller & Dixon (1960) prepared *N*-acetylcorticotropin by selective acetylation of the *N*-terminal serine residue of the natural hormone. When assayed for melanocyte-stimulating activity in isolated frog skin, acetylcorticotropin was found to be only about eight times more active than corticotropin itself, which meant that it was still from 10 to 20 times less active (on a molar basis) than α-MSH. Clearly an alteration to serine is only one of the factors involved, and the chain of amino acids from positions 14 to 39 must also adversely influence the potential melanocyte-stimulating activity of corticotropin. More direct evidence for the importance of the length of chain emerged when the free tridecapeptide amide, corresponding to the *N*-terminal tridecapeptide in corticotropin, was found to be appreciably more active (10–20 times) than corticotropin and about as active on a molar basis as *N*-acetylcorticotropin. Acetylation of the tridecapeptide amide led to the total synthesis of fully active α-MSH (Guttmann & Boissonnas, 1959; Hoffmann, K., unpublished results, 1959).

e. The Chemical Basis of Melanocyte-Stimulating Activity

α-MSH and β-MSH's both represent highly active structures and therefore the *N*-terminal, Asp.Glu.Gly.Pro (pig), Asp.Ser.Gly.Pro (ox), and Ala.Glu.Lys.Lys.Asp.Glu.Gly.Pro (man), and the *C*-terminal, Pro.Lys.Asp (common to all three), sequences in β-MSH's cannot be essential for melanocyte-stimulating activity, since in α-MSH they are replaced by *N*-acetylserine and valine amide, respectively. And similarly, it could be argued that acetylserine and valine amide are not specifically essential for the activity of α-MSH. What then is the minimum structure which is essential for melanocyte-stimulating activity?

A comparison of the structures of known melanocyte-stimulating substances suggests that the structure which is essential for biological activity resides in the sequence of amino acids which is common to all the active molecules (see fig. 1). Synthetic studies have shown that this is in fact the case. Thus the pentapeptide, His.Phe.Arg.Try.Gly (Hoffmann, Woolner, Spühler & Schwartz, 1958; Schwyzer & Li, 1958) possesses a small but nevertheless significant melanocyte-stimulating activity in frog skin, although it should be emphasized that the fragment of melanocyte-stimulating hormone is from 10^5 to 10^6 times less active than the natural hormone. The specific biological activity of active fragments increases progressively as the chain-length is increased (for example, the octapeptide, Ser.Met.Glu.(NH_2).-His.Phe.Arg.Try.Gly, is about 50 times more active than the pentapeptide (Hoffmann, Thompson & Schwartz, 1957)), and although the tridecapeptide amide is over 1,000 times more active than the octapeptide, it is still 20 times less active than the *N*-acetyl derivative corresponding to the natural hormone.

The question is where to draw the line. How active does a molecule have to be before it attains the status of a "hormone"? Clearly one must differentiate between structures which are shown to be active by in-vitro assay, and those which are also capable of replacing the natural hormone under the particular physiological conditions which exist in the whole animal. In the case of melanocyte-stimulating hormone this distinction is at present difficult to evaluate, since the function of this hormone in the mammal is not precisely known. However, the synthesis of active corticotropic peptides should lead to a much clearer evaluation of the relationships which exist between chemical structure and biological function, since the various biological activities of this hormone in the mammal are readily detected and measured. It may well emerge that even those parts of a hormone molecule which appear not to be specifically associated with its biological properties may nevertheless play other important roles, such as in protecting the active structure from inactivation so that it may survive being transported from the site of synthesis to its target organ elsewhere in the body; and in facilitating its attachment to specific sites within the target organ.

f. Species Differences among Melanocyte-Stimulating Hormones

Since melanocyte-stimulating hormone has no known function in mammals, the possibility exists that it may simply be a molecular relic of evolution. However, the fact that differences in amino-acid sequence have been found in melanocyte-stimulating hormones from different mammalian species suggests that mutations affecting its structure have occurred within mammals. If such mutations have in fact taken place, it would be surprising if the surviving ones are limited to those which result in the formation of an active hormone molecule, unless active melanocyte-stimulating hormone does indeed have survival value in mammals. The glutamic acid–serine interchange in pig and ox β-MSH's has occurred in a part of the molecule which is not essential for biological activity, and in this respect it is reminiscent of similar structural changes found in the unessential *C*-terminal portion of corticotropins. The occurrence of the additional *N*-terminal tetrapeptide, Ala.Glu.Lys.Lys, in human β-MSH appears to be another example of the same phenomenon.

On the other hand, when a change in structure occurs in a part of the molecule which in all probability is essential for high melanocyte-stimulating potency (such as the replacement of lysine by arginine in human β-MSH) it is significant that it involves the replacement of one amino acid by another which is chemically related to it (cf. vasopressins, section 1e). Thus, in spite of the considerable changes in structure which can occur elsewhere in melanocyte-stimulating molecules (see fig. 1), it appears that the part of the molecule which is essential for high melanocyte-stimulating potency has been preserved by natural selection. Presumably mutations which lead to chemically unrelated structural changes in essential parts of a hormone molecule do not survive because they result in the formation of molecules which no longer possess the necessary biological specificity and/or potency to function as the hormone under the prevailing physiological conditions.

The chemical studies which have been carried out on oxytocins, vasopressins, corticotropins, and melanocyte-stimulating hormones have enabled us to formulate certain empirical correlations between their chemical structures and biological properties. For example, it is established that a peptide hormone such as vasopressin, or corticotropin, defined in terms of a unique chemical structure, can possess more than one biological activity, and that these biological properties can be related to specific sequences of amino-acid

residues in their molecules. The confusions and contradictions which have been inherited from past studies on grossly impure hormone preparations are beginning to be resolved, and now that chemically defined and reproducible hormone preparations have become available it is to be hoped that future studies will add to our knowledge of the mechanisms by which they act, and of the sequence of biochemical events which they catalyse in the living organism.

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METHODS OF INSULIN ASSAY

G. A. STEWART B.Sc. A.R.I.C.

Biological Control Laboratories
The Wellcome Foundation, London

1 Assay of insulin for clinical use
  a Rabbit blood-sugar method
  b Mouse convulsion method
  c Demonstration of the prolonged action of modified insulin preparations
2 Assay of minute amounts of insulin
  a In-vivo methods
  b In-vitro methods
3 Chemical assay of insulin
  a Fibril method
  b Chromatographic method
4 Comparison of the methods
References

The discovery of insulin made it necessary to devise methods for assessing its activity. Assays based on hypoglycaemia in rabbits and convulsions in mice were soon established and are used to control pharmaceutical preparations of insulin. The determination of insulin in blood, however, requires more sensitive methods; the isolated diaphragm and epididymal fat of the rat, and rats and mice from which endocrine glands antagonistic to the action of insulin have been removed, are employed for this purpose. Specific chemical methods of assay have been sought; those based on the formation of insulin fibrils and on paper chromatography show promise.

1. Assay of Insulin for Clinical Use

a. Rabbit Blood-Sugar Method

Banting, Best, Collip, Macleod & Noble (1922) found that insulin-induced convulsions in rabbits were usually associated with a blood-sugar concentration of 45 mg./100 ml. The amount of insulin which produced this concentration in 2–4 hours was used as a unit of activity. Later (League of Nations, 1926), the unit was defined as one-third the amount required to produce this effect in a rabbit starved for 24 hours. The method was unsatisfactory, since rabbits vary considerably in their sensitivity to insulin. Marks (1925) recommended the comparison of one preparation in terms of another in two similar groups of rabbits; one group received the preparation of known activity, the second the preparation of unknown activity. On another day the procedure was reversed to complete a "cross-over" test. By averaging the hypoglycaemic responses to each preparation over both days, the influence of the variation in sensitivity between rabbits was considerably reduced and an estimation using the variable "rabbit unit" of activity was avoided. In a later modification (Marks, 1936) two such cross-over tests were performed, using two dose-levels of both the standard and unknown preparations to provide an additional check on the potency.

Since 1925 when the First International Standard for Insulin was adopted which, based on the rabbit unit, was said to contain 8 units/mg. of activity (League of Nations, 1926), all preparations of insulin have been assayed directly or indirectly in terms of it.

All subsequent investigations of the rabbit blood-sugar method have employed cross-over designs. The reduction in blood sugar, the final concentration, the reduction or final concentration as percentages of the initial value, either at a given time after insulin, or averaged over a period of time, have been used as metameters for the estimation of insulin activity. They exhibit a linear regression on log dose of insulin.

Culhane (1928) compared the hypoglycaemic response of rabbits to insulin every hour for five hours and found that the average percentage reduction in blood sugar up to two hours gave results comparable to those based on the average response over five hours. Blood-sugar levels determined initially (Pugsley & Rampton, 1948), one hour (Lacey, 1946), and five hours (Pugsley & Rampton, 1948) after the subcutaneous injection of insulin contributed little to the precision of the assay. The determination of blood sugar 50 minutes after the intravenous injection of insulin, by itself (Young & Romans, 1947) gave results as precise as those based on the average response one-and-a-half and three hours or two and four hours after subcutaneous injection of insulin (Pugsley & Rampton, 1948).

Lacey (1941) used a procedure adopted by the *Pharmacopeia of the United States of America* (1942) (USP XII) in which the unknown, assumed at three different potencies, was assayed in rabbits in terms of one dose of standard in three separate and complete cross-over tests. The average percentage reduction in blood sugar during five hours with one of the test dilutions was divided by the corresponding value for the standard, which when multiplied by 100 gave an index of activity. Indices obtained for the three test dilutions were plotted against the assumed potencies. The potency of the unknown was that corresponding to an index of 100 (*Pharmacopeia of the United States of America* (1942) (USP XII)). The method is limited, however, by the need for the assumed potencies to bracket closely the true potency (Lacey, 1946).

The fall in blood sugar after insulin, and this value expressed as a percentage of the initial concentration, are correlated with the initial blood-sugar concentration (Hemmingsen & Marks, 1932). Modern statistical procedures eliminate this bias by covariance analysis. In addition, where (2+2) or (3+3) cross-over designs are employed, the precision of the assay can be calculated after errors due to variation in rabbits, groups and days have been eliminated (Bliss & Marks, 1939a, 1939b; Fieller, Irwin, Marks & Shrimpton, 1939a, 1939b; Smith, Marks, Fieller & Broom, 1944). (For a critical review of these various statistical procedures see Smith (1950).)

Lacey (1946) showed that the dilution of a solution of insulin before injection may affect the subsequent hypoglycaemic response. He suggested that a solution for clinical use should therefore be injected undiluted at two dose-levels and compared with a similar solution of the standard which was also injected undiluted, since the presence of an impurity which might modify the rate of action would be more likely to be detected in the undiluted solution, and the response more likely to reflect the clinical response. The procedure is adopted for the examination of the insulin preparations with prolonged action.

b. Mouse Convulsion Method

Small doses of insulin injected into mice produced convulsions (Fraser, 1923), and the percentage of mice responding increased with increase in dose (Hemmingsen & Krogh, 1926; Trevan & Boock, 1926). The metameter obtained by converting percentage response to normal equivalent deviation was linearly related to log dose (Hemmingsen, 1933b). Weighting coefficients were applied, the highest being given to 50% response; responses at the extreme ends of the dose-response curve were given the lowest values because of the greater range in doses producing them (Marks, 1940).

Many factors influence the response of mice to insulin. Increase in assay temperature from 24° C. (Sellar & Smart, 1959) or 29° C. (Hemmingsen & Marks, 1933) to 37° C. increased the sensitivity of mice to insulin without affecting the slope (*b*) of the dose-response curve. Mice stored for one week before assay at 6–13° C. produced a higher slope (*b*) but were less sensitive than mice stored at 27–30° C., even though the assay temperature (34° C.) was the same (Sellar & Smart, 1959). A period of starvation between 4 and 22 hours did not alter the response of the mice to insulin (Trevan & Boock, 1926). Mice fed on bread and water and starved overnight were more sensitive to insulin and yielded a higher slope than mice fed a diet containing less carbohydrate and more protein and fat (Rowlinson & Lesford, 1948). With mice whose weights varied over a range of 1.5 g., the slope increased significantly as the weight of mice increased from 14.5 g. to 22 g. (Young & Stewart, 1952); the relationship no longer existed when the mice were used again. With weights varying over a range of 4 g. (Hemmingsen, 1933a) or of 5 g. (Smith, 1950) there was no correlation between the slope and body-weight. Subcutaneous injection of promethazine hydrochloride (50 μg./mouse) 30 minutes before insulin increased the sensitivity of mice approximately threefold without affecting the precision of the assay (Stewart & Smart, unpublished data, 1952). Mice fed on bread and milk and starved overnight when treated in this way permitted a direct assay of insulin in tissue extracts containing ethanol, since their greater sensitivity (2–4 milli-units (mU.)) allowed the extracts to be diluted to such an extent that any interfering effects of the ethanol were eliminated.

When insulin is assayed by subcutaneous injection into mice, glucagon does not interfere (Zimmerman & Donovan, 1948; Volk & Lazarus, 1959). In the assay of insulin for clinical use, where the quality of the insulin is equivalent to that of the standard, it is sufficient to use a (2+2) assay design (*British Pharmacopoeia*, 1958a). Four groups of 24 mice with weights varying over a range of 5 g. are used, which receive respectively subcutaneous injections of two dilutions of the standard and two of the test preparation. The mice, after injection, are observed for 90 minutes at a uniform temperature (29–35° C.). The numbers of mice which die, exhibit frank convulsions, or which lie still for more than 2–3 seconds when placed on their backs, are recorded. The result is calculated by standard statistical methods (Finney, 1952).

In an endeavour to overcome possible observer error in determining positive responses, mice, after injection with insulin, were placed on a wire-mesh screen inclined at an angle of 60° (Thompson, 1946; Stephenson, 1959) or placed within a rotating cylinder similarly inclined (Young & Lewis, 1947). Mice exhibiting convulsive symptoms were unable to retain their foothold and fell from the screen or cylinder and were counted as positive responders. Compared with the results from the method using continuous observation, no increase in precision resulted.

With doses of insulin between 1.6 units and 40 units/10 g. mouse, the period between injection and convulsion increased with increase in dose (Satke-Eichler & Stumpf, 1955). The effect, however, does not appear to depend on a direct hypoglycaemic response, and the method has little practical application because of its insensitivity.

c. Demonstration of the Prolonged Action of Modified Insulin Preparations

The *Pharmacopeia of the United States of America* (1955) (USP XV) combines in a rabbit cross-over test a measure of the potency of globin zinc insulin (GZI) and a demonstration of its characteristic action. The undiluted sample is injected subcutaneously into one group of rabbits at a potency assumed to be equal to that of a standard prepared from USP "Globin and Insulin Reference Standards". The standard is injected subcutaneously into a similar group. A cross-over test is performed on at least 30 rabbits. The blood-sugar curve obtained with the unknown must match that of the standard within narrow limits. Protamine zinc insulin (PZI) is examined similarly, using a protamine reference standard.

In Great Britain at a time when a freeze-dried GZI standard was prepared (British Insulin Manufacturers' Biological Sub-Committee, 1952), no statistically significant prolonged action of GZI in rabbits could be demonstrated when it was compared with unmodified insulin unless an impracticably large number of animals was used. It was therefore recommended that GZI should be assayed against the GZI standard by the mouse convulsion method (*British Pharmacopoeia*, 1953).

Stewart & Smart (1953) found that the guinea-pig was more sensitive to qualitative differences in insulin preparations than was the rabbit, a significant prolonged action being obtained in a single one-day test if GZI was injected subcutaneously into six guinea-pigs and the action compared with that of the same dose of unmodified insulin injected into a similar group. This method may also be used for the demonstration of the prolonged action of PZI, isophane insulin, and the insulin zinc suspensions (*British Pharmacopoeia*, 1958b).

2. Assay of Minute Amounts of Insulin

a. In-Vivo Methods

i. Rats

The normal animal is generally too insensitive for the assay of insulin-like activity in blood. When the hypophysis or adrenals are removed, the sensitivity of an animal to insulin is increased, and when alloxan is injected variations in response to injected insulin, caused by the presence of endogenous insulin, may be reduced. Animals prepared in this way were used in attempts to assay insulin-like activity of blood.

Gellhorn, Feldman & Allen (1941) found that the rat with both hypophysis and adrenals removed is 16 times more sensitive to insulin than with only one endocrine source removed. A straight line was found to relate the blood-sugar concentration and the log of the dose of insulin over the range 0.3–1.0 mU. insulin/100 g. of body-weight. Rats from which the adrenal medullae had been removed, injected with alloxan and then hypophysectomized, showed a similar regression of reduction in blood sugar after insulin on log dose (0.125–5.0 mU./rat, approximately 200 g.) (Anderson, Lindner & Sutton, 1947). Hypophysectomized rats with diabetes induced by

alloxan, when given glucose orally before an injection of insulin in 5 % human albumin, showed a linear regression of reduction in blood sugar on log dose (2.5–10 mU./rat) (Beigelman, Goetz, Antoniades & Thorn, 1956). The albumin was said to reduce the variability in response in different rats, and increased their sensitivity to insulin.

Bornstein (1950) carried out total adrenalectomy in the hypophysectomized rat with diabetes induced by alloxan (ADHA rat). The animals had access to an unlimited amount of food and were maintained at 22–26° C. up to the time of use. The assay was carried out at 37° C. After one hour's fast to stabilize blood-sugar concentrations, the animals were injected subcutaneously with insulin and the blood sugar determined one hour later. The assay was also carried out at 27° C. on similar rats after one-and-a-half hours' fast (Bornstein & Lawrence, 1951a, 1951b). A linear relationship obtained between reduction in blood sugar and log dose of insulin (0.05–0.5 mU./rat, approximately 180 g.). With this method, samples of human plasma obtained two hours after oral glucose were found to possess insulin-like activity equivalent to 0.19–0.29 mU./ml. (Bornstein & Lawrence, 1951a).

The sensitivity of the ADHA rat to insulin is not affected by exogenous cortisone, growth hormone or adrenocorticotropic hormone (Bornstein & Lawrence, 1951b). Nevertheless there exists in the plasma of some diabetics an insulin antagonist which interferes with the assay, since rats receiving this plasma and subsequently injected with insulin showed a marked reduction in sensitivity (Bornstein & Lawrence, 1951b; Bornstein & Trewhella, 1951).

ii. Mice

Mice are usually more sensitive than rats to insulin. In the hypophysectomized alloxan-diabetic mouse there was a linear regression of the fall in blood-sugar concentration on log dose of insulin 20 minutes after intravenous injection (0.25–4.0 mU./mouse) and 30 minutes after intraperitoneal injection (1.0–4.0 mU./mouse) (Anderson, Wherry, Bates & Cornfield, 1957). When the intraperitoneal route was used, the mice were less sensitive to insulin but the precision of the assay was greater (λ[1] = 0.37–0.48) than with the intravenous route (λ = 0.68–0.76). Since saline produced a fall in blood sugar, control animals should always be included. It is doubtful whether the method is sensitive enough for the assay of insulin-like activity in plasma.

Baird & Bornstein (1959) used adrenalectomized alloxan-diabetic mice anaesthetized with sodium pentobarbitone. Insulin injected intravenously into mice with initial blood-sugar concentrations between 130 mg./100 ml. and 280 mg./100 ml. produced a fall in blood sugar which was linearly related to log dose (1–4 mU./25 g. mouse). The method was applied to the determination of insulin-like activity of plasma extracts. Mice so treated exhibited anaphylaxis and could therefore be used only once.

b. In-Vitro Methods

i. Rat Diaphragm

Insulin increases the glycogen content of the isolated hemidiaphragm of the rat bathed in a medium containing glucose (Gemmill, 1940). Measuring the glycogen content of quarter diaphragms, Perlmutter, Weisenfeld & Mufson (1952) failed to detect insulin-like activity of human serum. Since the lowest concentration of insulin used to obtain a response was 5 mU./ml., the method lacked sensitivity. Groen, Kamminga, Willebrands & Blickman (1952) made a detailed study of the relationship between the concentration of insulin and the glucose uptake. Hemidiaphragms from starved rats weighing 80–100 g. were washed in ice-cold buffer (Gey & Gey, 1936). One hemidiaphragm from each of four rats was placed in a flask with 2 ml. of ice-cold buffer containing 2 mg./ml. glucose. The other four hemidiaphragms were placed in a similar flask. Both flasks were gassed with O_2+CO_2 (93:7, v/v) (pH 7.4). Insulin was present in one of the flasks. After incubation, with shaking, at 37° C. for 90 minutes, the glucose content of each flask was determined. The difference in glucose utilization/100 mg. of wet diaphragm between the insulin-treated tissues and the control was a measure of the insulin effect. A concentration–response curve was constructed for insulin between 0.0001 unit/ml. and 1.0 unit/ml. The effect was maximal at 100 mU./ml. and sensitivity varied from 0.001 to 1 mU./ml., depending on the time of year.

Vallance-Owen & Hurlock (1954), Randle (1954, 1956) and Willebrands, van der Geld & Groen (1958), using procedures similar to that of Groen *et al.* (1952), studied the method. Hemidiaphragms from albino rats of similar body-weight, none exceeding 150 g., were used singly. Various metameters were employed as a measure of the insulin effect. The uptake of glucose over 90 min./100 ml. buffer/10 g. dry weight of tissue increased with increase in insulin concentration, 0.01–1.0 mU./ml. (Vallance-Owen & Hurlock, 1954). Over a 3-hour period the cube root (mg. glucose taken up/g. wet diaphragm/hr.) was linearly related to log insulin concentration, 0.125–32 mU./ml. (Randle, 1954). Correcting the glucose uptake for the dry weight of diaphragm did not decrease the variation in response between tissues (Randle, 1956). The glucose uptake/hemidiaphragm gave a smaller standard deviation than the uptake corrected for tissue weight; an approximately linear relationship existed between metameter and log insulin concentration, 0.03–8 mU./ml. (Willebrands *et al.* 1958). The index of precision (λ) varied from 0.18 to 0.74 (Randle, 1956).

In applying the method to the assay of insulin-like activity of plasma, Vallance-Owen & Hurlock (1954) used undiluted plasma and constructed for each assay a standard curve with three concentrations of insulin from which the activity of the plasma was calculated. The values for plasma from normal subjects were < 1 mU./ml. Randle (1954) diluted samples of plasma with buffer (1 in 4 and 1 in 16) and conducted (2+2) assays against two insulin solutions similarly diluted, and found approximately 13 mU./ml. These differences are due to dilution of the plasma with buffer, as the effects of substances which antagonize the action of insulin on the diaphragm are reduced by dilution. The principal substance measured in plasma is likely to be insulin, since cysteine, which inactivates insulin, abolishes the insulin-like activity of plasma (Randle, 1954; Vallance-Owen & Hurlock, 1954). Differences in adrenaline concentration between diaphragms may account for their variation in sensitivity to insulin: Groen, van der Geld, Bolinger & Willebrands (1958) found that the addition of 0.1 μg./ml. of adrenaline significantly reduced the effect of 0.5 mU./ml. insulin on glucose uptake.

Manchester & Young (1958) showed that insulin increases the incorporation of radioactivity from [^{14}C]amino acids into

[1] The index of precision, λ, $= s/b$ where s is the standard deviation of points about the regression line of slope, b.

rat diaphragm in the absence of glucose. A method based upon this observation was described (Manchester, Randle & Young, 1959) whereby the amount of [^{14}C]glycine incorporated into diaphragm protein in the presence of various doses of insulin was measured. The method is sensitive to 0.05 mU./ml. of insulin (λ = 0.4–0.5).

ii. Rat Epididymal Fat

Insulin enhances glucose uptake, oxidation of glucose to CO_2 and incorporation of glucose carbon into ether-soluble lipid in the epididymal fat pad of the rat maintained in Krebs-Henseleit buffer (Winegrad & Renold, 1958). An assay sensitive to 10 μg./ml. insulin, based on these effects, has been devised by Martin, Renold & Dagenais (1958). The fat pad of a fed male Wistar rat was cut into three or six equal pieces, and each piece placed in a separate vessel in buffer containing gelatin, [1-^{14}C]glucose, either with or without appropriate dilutions of insulin. The vessels, gassed with $O_2 + CO_2$ (95 : 5, v/v), were incubated at 37.6° C. for two hours in a Dubnoff shaker. The $^{14}CO_2$ liberated was converted to $Ba^{14}CO_3$ and counted. The difference in readings between flasks containing insulin and no insulin, expressed as counts/min./total CO_2/100 mg. fat, was a measure of the insulin effect. There was a linear relationship between the square root of this metameter and log concentration of insulin (0.031–0.5 mU./ml.; λ = 0.31). Alternatively the insulin effect was measured by determination of glucose uptake. Ball, Martin & Cooper (1959) simplified the method. In the absence of insulin more gas is consumed by the tissue than is released. Insulin increases the net gas exchange/unit weight of fat, the effect being proportional to concentration. To eliminate variation in response between different pieces of fat the initial response to insulin is expressed as a percentage of the subsequent maximum response to a large dose of insulin. The addition of gelatin increased the insulin effect, possibly by preventing insulin from being lost through adsorption on to the glass. Epididymal fat from rats weighing 150–250 g. was most suitable (Hagen, Ball & Cooper, 1959), and the fat from albino rats was more sensitive to insulin (approximately 10–25 micro-units/ml.) than that from Norwegian hooded rats (approximately 250 micro-units/ml.) (Stewart & Neville, unpublished data, 1959). A comparable difference in insulin-sensitivity has been obtained with diaphragms of these two strains of rat (Randle, 1956).

iii. Other In-Vitro Methods

Krebs & Eggleston (1938) found that insulin increased the oxygen consumption of minced pigeon breast muscle in phosphate buffer. The increase was proportional to the concentration of insulin (10–1,000 mU./ml.). The effect was claimed to occur only with zinc-free insulin, but Shorr & Barker (1939) obtained it with crystalline zinc insulin (22 units/mg.). Since Krebs and Eggleston used insulin containing only 17–20 units/mg., some impurity may have enhanced the inhibitory action of zinc.

Woods, Wight, Hunter & Burk (1953) found that if a melanoma maintained in mice was sliced and incubated anaerobically at 38° C. in a suitable medium, the amount of lactic acid produced expressed as CO_2/mg. dry weight tissue/hr. increased if insulin was present in a concentration of 4–100 mU./ml. Glucagon did not interfere with the action, whereas zinc did.

Sliced abdominal mammary tissue from Norwegian rats in the 12th–15th day of lactation, when maintained in Krebs bicarbonate medium, showed in the presence of insulin an increase in net gas output (Balmain, Cox, Folley & McNaught, 1954). The increase in μl. CO_2/mg. final dry weight of tissue/hr. from 30 minutes to 150 minutes was linearly related to log concentration of insulin (approximately 0.01–5.0 units/ml.). This tissue is less sensitive than rat diaphragm (Randle, 1956), but the method is probably more precise (λ = 0.18–0.28).

Insulin also had a quantitative action on chick heart explants, from 13-day-old embryos, grown in vitro for six days (Leslie & Paul, 1954). There was a linear relationship between glucose uptake and log insulin concentration between 0.1 mU./ml. and 1,000 mU./ml., and the increase in ribonucleic acid phosphorus and deoxyribonucleic acid phosphorus both showed a linear relationship on log insulin concentration between 10 mU./ml. and 1,000 mU./ml.

None of these procedures has been generally adopted for the assay of insulin, possibly because they are relatively insensitive and because suitable tissue would either be expensive or difficult to maintain.

3. Chemical Assay of Insulin

Many of the chemical methods described for the assay of insulin are based on non-specific protein reactions. However, two others appear more promising.

a. Fibril Method

The precipitate formed when a solution of insulin in dilute acid is heated (Blatherwick, Bischoff, Maxwell, Berger & Sahyun, 1927) is composed of radially oriented fibrils (Waugh, 1946). An assay based on this property was devised (Waugh, Thompson & Weimer, 1950). An acid solution of insulin was repeatedly heated and frozen until intense flow birefringence occurred. The fibrils so formed were broken by expulsion through a hypodermic needle, and used to seed the standard and test solutions of insulin. These solutions were stirred continuously overnight at 48° C. and the precipitate formed was collected, washed, dried and weighed. The weight of fibrils obtained was proportional to the amount of insulin present. Pig, sheep and ox insulin when inoculated with seed prepared from pig insulin gave quantitative recoveries, whereas seed prepared from ox insulin (Waugh *et al.* 1950) or from sheep insulin (Foster, Macdonald & Smart, 1951) did not, unless considerable agitation was employed (Grodsky, 1958). The method could not be applied to samples containing less than 11 units/mg. or to solutions of GZI (Foster *et al.* 1951). When applied to PZI and solutions of unmodified insulin which had lost activity under adverse conditions of storage, the results were equivocal (Waugh *et al.* 1950; Foster *et al.* 1951; Kärnbach & Bilk, 1954).

Grodsky (1958) applied the method to the assay of insulin in small samples of pancreas. The fibrils were either re-suspended in acid solution and the optical density determined, or dissolved in boiling NaOH, and the tyrosine content determined colorimetrically.

b. Chromatographic Method

Robinson & Fehr (1952) assayed the insulin content of PZI by paper chromatography. A solution of insulin was streaked across a paper and the chromatogram developed, with the upper phase of *n*-butanol, glacial acetic acid and

water (3 : 1 : 4 by volume). The insulin spots were stained with bromocresol green, the colour intensified in ammonia vapour and eluted with borate buffer and compared with that of a similarly treated insulin standard. A linear relationship existed between absorption and insulin concentration. A similar technique using bromophenol blue has been described (Grodsky & Tarver, 1956) which is generally too insensitive for the assay of insulin in pancreas (Grodsky, 1958).

Fenton (1959) separated insulin from associated impurities by ascending chromatography with the upper phase of butanol-2-ol, 1% acetic acid (1 : 1, v/v). Bromocresol green in acetate buffer was used to locate the insulin, the colour intensified in ammonia vapour, eluted with 0.1 N-NaOH in 95% ethanol (1 : 1, v/v) and read in the absorptiometer. The extinction so determined was linearly related to the weight of insulin applied to the paper, 0–110 μg. Sensitivity is about 0.5 unit. Potencies obtained for samples of crystalline and amorphous insulin and for solutions which had lost potency under unfavourable conditions were in good agreement with those obtained by the mouse convulsion method.

Circular chromatography has been used (Bouman & Homan, 1958), because it gives a sharper separation between insulin and glucagon; but the sensitivity of the method (approximately 12.5 units) is low and cannot be applied to insulin containing less than 19 units/mg.

4. Comparison of the Methods

The rabbit blood-sugar method is less sensitive than the mouse convulsion method, usually requiring doses greater than 0.1 unit/kg., as compared with less than 30 mU./mouse. The rabbit method, however, is more precise, since in assays of the Third (Miles, Mussett & Perry, 1952) and Fourth (Bangham & Mussett, 1959) International Standards for Insulin, the average statistical weight[2] per animal appearance was 9.5 or less, and 18.4 respectively for rabbits in a twin cross-over assay, and only 3.2 and 2.6 respectively for mice. Three assays, each using 96 mice, gave approximately the same statistical weight as 24 rabbits in the cross-over assay (Bangham & Mussett, 1959), but were completed more quickly. With insulin for clinical use, potencies obtained by both methods are similar. However, in the assay of the Second International Standard for Insulin against the First (League of Nations, 1936), where the quality of the two standards was markedly dissimilar, the potency obtained in rabbits was lower than that obtained in mice, probably owing to different rates of absorption of each preparation in the two species.

The two chemical methods described are less sensitive, probably less specific, but more precise and less costly than the biological methods. The fibril method using gravimetric procedures requires a large volume of solution containing at least 1 unit/ml.; using absorptiometric techniques, it may detect about 0.5 unit/g. of pancreas. Grodsky (1958) found that the method was not highly specific, since fraction *B* from insulin, esterified insulin and carboxypeptidase-inactivated insulin also formed fibrils. The chromatographic method will estimate between 0.5 unit and 12.5 units of insulin. Impurities associated with low-grade insulin may interfere. Fenton (1959) calculated that a single determination of potency by his method was equal in precision to that obtained with 960 mice.

Of the other in-vivo methods, only that using the ADHA rat is sensitive enough to estimate the insulin-like activity of undiluted plasma; the less sensitive methods may be used to assay fractions from plasma. While the precision of these in-vivo assays varies widely (λ = 0.31–0.76) and is of the same order as that of the in-vitro assays using the diaphragm or epididymal fat of the rat (λ = 0.18–0.74), the preparation of the animals is lengthy and requires considerable skill. The assay based on the oxidation of [1-^{14}C]glucose to $^{14}CO_2$ in rat epididymal fat is extremely sensitive (0.01 mU./ml.). Methods measuring glucose uptake are reproducible, but the minimum amount of insulin may vary from 0.01 mU./ml. to 2.0 mU./ml. With the possible exception of the method measuring the incorporation of [1-^{14}C]glycine in diaphragm protein, the methods for the assay of plasma insulin are non-specific, as the presence of insulin antagonists and of possible synergists and insulin-like substances only permits the measurement of effective insulin-like activity.

[2] The "statistical weight" of an assay is the reciprocal of the variance.—ED.

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METABOLISM OF PEPTIDE HORMONES

A. J. KENNY M.B. B.S. Ph.D.

Department of Biochemistry
University of Leeds

1 Metabolism of insulin
  a In-vitro investigation of the insulin-inactivating and insulin-degrading systems
  b Liver perfusion studies
  c In-vivo studies
2 Metabolism of glucagon
  a In-vitro investigation of the glucagon-inactivating and glucagon-degrading system
  b In-vivo studies
3 Metabolism of some other peptide hormones
4 Conclusions and summary
References

It may be taken for granted that the magnitude of action of a hormone is determined by the concentration of effective hormone at its site of action. This effective local concentration depends upon a number of factors, which includes the rate of arrival of the hormone at its site of action, and of any circulating antagonists of it, and the rate of removal of the hormone and its antagonists from the site of action. A simple method of removal of the hormone obviously can be by its metabolic inactivation and degradation. This chapter is concerned with the inactivation and degradation in tissues of some of the peptide hormones. Antagonists of such hormones are not considered here (see, however, Vallance-Owen, 1960). There is evidence that the catabolism of peptide hormones is important in limiting their duration of action, particularly that of those with an ephemeral effect.

Several peptide hormones have been shown to be inactivated by tissues. This can be demonstrated by incubating the hormone with a tissue or tissue extract and noting the loss in biological activity. In-vitro experiments of this sort enable the mechanism of inactivation to be studied in a relatively simple system. It is also possible to observe inactivation of a hormone in the intact animal either by inference from the way in which the effect diminishes with time, or more directly, by the recovery of the administered hormone from the animal's carcass and organs. The former technique is open to the criticism that a single observed effect may be the result of a multiplicity of factors in addition to the concentration of the hormone under investigation. The more direct method usually requires the administration of large doses in order to permit bio-assay of the recovered hormone. It is possible that unphysiological doses are disposed of by channels which do not usually operate.

A different approach to the study of the metabolism of peptide hormones has been made possible by the use of radioisotopes. The most common technique is to iodinate the peptide with iodine containing the radioisotope ^{131}I, with or without carrier iodine ^{127}I. Iodination of peptides and proteins occurs mainly by substitution in the 3- and 5-positions of the tyrosine residues. The product is therefore a new compound, chemically different from the native hormone. Since extensive iodination may reduce biological activity, it is usual to limit the extent of iodination to a level at which biological activity is not measurably impaired—e.g., one, or less than one, atom of iodine per molecule of peptide. Other radioactive labels the introduction of which does not involve a chemical transformation of the hormone are possible—e.g., sulphur (^{35}S) or tritium (^{3}H). But the introduction of these labels presents problems either of biosynthesis or of assay which are avoided by the use of ^{131}I.

[^{131}I]Iodohormones have been incubated in vitro with isolated tissues, perfused through isolated organs and injected into intact animals. In each case it has become conventional to follow the separation of radioactivity into either the precipitate or supernatant after trichloroacetic acid (TCA) has been added to the tissue or fluid. TCA-soluble (supernatant) radioactivity is attributed to radioactive degradation products of the hormone. TCA-insoluble (precipitate) radioactivity is assumed to include intact labelled hormone.

It is also possible to supplement or replace bio-assays and radioisotopic assays by a chemical determination of the degradation of the hormone. Chemical methods for the determination of the hormone itself are not sufficiently specific to be of any use. But it is relatively simple to follow proteolysis of peptides by such methods as the increase in non-protein nitrogen, ninhydrin colour, or absorption at 280 mμ. Such methods are applicable only to in-vitro experiments in which relatively large amounts of pure hormone are used.

1. Metabolism of Insulin

Interest in the fate of insulin arose shortly after its isolation as a crystalline protein. Schmidt & Saatchian (1929) reported that breis prepared from several rabbit tissues could inactivate insulin by what was presumed to be a proteolytic enzyme system. Lehmann & Schlossmann (1939) showed that a cell-free muscle extract, which inactivated insulin, contained two components capable of acting separately. One was a heat-stable and diffusible factor. The other was heat-labile and enzymic in nature. It was proposed that both factors inactivated insulin by reduction and cleavage of disulphide bridges. The relative importance of these two possible processes—reduction and proteolysis—is still one of the subjects of recent investigations.

Most of the now extensive published work on the inactivation and degradation of insulin has appeared in the past ten years. Mirsky & Broh-Kahn (1949) proposed the term "insulinase" for the enzyme that inactivates insulin. While the term is convenient, it seems preferable to avoid it at present since it implies the existence of a single enzyme of high specificity towards insulin. Neither point can be regarded as substantiated at present. Other terms will be used—"insulin-inactivating system" or "insulin-degrading system"—the former when biological inactivation is considered, the latter when degradation of insulin iodinated with ^{131}I ([^{131}I]iodoinsulin) is studied.

The insulin-inactivating and insulin-degrading systems are widely distributed among tissues. In the rat most activity is found in the liver, but kidney and muscle are also active. Brain is weakly active, plasma and red cells are inactive (Mirsky & Broh-Kahn, 1949). A pancreatic enzyme, which

has been separated as a homogeneous protein from elastase, will also degrade [^{131}I]iodoinsulin (Lewis & Thiele, 1957). Since liver has the greatest activity in attacking insulin, most of the work has been done with this tissue. Livers from many sources—rat, mouse, guinea-pig, rabbit, cow, chicken, duck, bull-frog, monkey and man—have been shown to contain inactivating or degrading systems (Mirsky, 1957; Weisenfeld, Jauregui & Goldner, 1957). The metabolism of insulin has been studied in vitro, in perfused organs and in vivo.

a. In-Vitro Investigation of the Insulin-Inactivating and Insulin-Degrading Systems

i. Properties of the Systems

The presence of heat-stable and heat-labile components in liver has been confirmed by biological assay (Mirsky & Broh-Kahn, 1949) and with [^{131}I]iodoinsulin (Mirsky, Perisutti & Dixon, 1955; Narahara, Tomizawa, Miller & Williams, 1955). In different experimental conditions the contribution of the heat-stable factor has varied from 2.5% to 30% of the insulin degraded by the combined action of both factors in unheated tissue preparations. The original presumption that the heat-stable factor was a sulphydryl compound (Lehmann & Schlossmann, 1939) is supported by the finding that oxidized glutathione and *p*-chloromercuribenzoate will inhibit degradation of [^{131}I]iodoinsulin by heated liver extracts. Reduced glutathione in low concentration at neutral pH values will degrade [^{131}I]iodoinsulin in the absence of any enzyme preparation (Narahara, Tomizawa & Williams, 1956). It is of interest that a purely reductive chemical process can lead to the formation of TCA-soluble radioactivity from [^{131}I]iodoinsulin.

Most attention has been focused on the heat-labile, enzymic component which, in unheated tissue extracts, accounts for most of the insulin destroyed. Some of the properties of the enzyme system in liver are shown in Table I. The results shown have been obtained with crude enzyme preparations. Recently Tomizawa & Halsey (1959) have isolated from ox liver a homogeneous enzyme which degrades [^{131}I]iodoinsulin. An enzyme catalysing the reduction of insulin (as determined by titration with *p*-chloromercuribenzoate) has been partially purified by Narahara & Williams (1959).

ii. Inhibitors of the Systems

Apart from the inhibitors which react with —SH groups, mentioned above, many other compounds have been found to produce inhibition of the destruction of insulin. Some of the main groups of inhibitors will be mentioned below.

Proteins and peptides. Insulin itself will inhibit the degradation of trace amounts of [^{131}I]iodoinsulin. This is only to be expected if the two substances are indistinguishable as substrates for the enzyme system. A few other proteins will also inhibit the degradation of [^{131}I]iodoinsulin: glucagon, corticotropin, α-casein, and somatotropin (Tomizawa & Williams, 1955). Some specificity in this effect exists, since other proteins tested, e.g., ribonuclease, α-lactalbumin and serum albumin, were not inhibitory at similar molar concentrations. Nevertheless, some of these proteins, together with a number of others, will exert an inhibitory effect if subjected to partial hydrolysis by acid or by proteolytic enzymes (Mirsky, 1957). Complete hydrolysis destroys this inhibitory action, which has therefore been attributed to the presence of certain peptides in the hydrolysates.

Another inhibitor, also thought to be a peptide, is present in liver and has been referred to as "insulinase inhibitor" by Mirsky, Simkin & Broh-Kahn (1950). It acts as a competitive inhibitor in vitro (Mirsky, 1957).

Amino acids and amino-acid derivatives. Among a number of amino acids tested, cystine, isoleucine, tryptophan, and 3 : 4-dihydroxyphenylalanine were found to be inhibitors. Several amino-acid derivatives, particularly those of L-tryptophan, have a similar action (e.g., kynurenic acid, indole-3-acetic acid, acetyltryptophanylethyl ester and ergothionine). Some of the derivatives have been shown to be competitive inhibitors, e.g., acetyltyrosine, phenylbutyramide, phenylpropionate (Mirsky, Diengott & Perisutti, 1956; Williams & Berg, 1956; Williams, Martin, Henley & Swanson, 1959).

Sulphonylureas. Several of the hypoglycaemic sulphonylureas have been shown to inhibit the degradation of [^{131}I]iodoinsulin in vitro (Mirsky, Perisutti & Diengott, 1956; Williams & Tucker, 1956; Volk, Goldner, Weisenfeld & Lazarus, 1957; Williams *et al.* 1959). The inhibition is non-competitive in type and the inhibitory concentration appears to be above the concentration likely to be achieved in the liver by therapeutic dosage in vivo. It is therefore very improbable that the inhibitory effect contributes significantly to the hypoglycaemic action of these compounds.

Other substances. Various other substances possess inhibitory action. These include several substances that regulate plant growth (auxins), some uncharacterized plant extracts, thyroxine, adrenalin, benzylpenicillin, chlortetracycline, phenethylbiguanide and decamethylenediguanidine (synthalin A) (Mirsky, 1957; Williams *et al.* 1959).

iii. Nature and Specificity of the Enzyme System Involved

Knowledge of the structure of insulin suggests two likely chemical processes by which it might become inactivated in tissues: cleavage of disulphide bridges and cleavage of peptide bonds. It has long been known that sulphydryl compounds, e.g., glutathione and cysteine, will reduce the S—S bridges of insulin (du Vigneaud, Fitch, Pekarek & Lockwood, 1931). The reaction can take place at neutral pH values, and biological inactivation is complete long before all the S—S bonds are reduced (Lens & Neutelings, 1950). Narahara & Williams (1959) have investigated the reduction of insulin by a rat-liver enzyme system. There is evidence that the reduction of insulin involves two distinct systems. One is glutathione reductase, requiring reduced triphosphopyridine nucleotide, which serves to regenerate reduced glutathione from the oxidized form. The other is an insulin-reducing system which requires reduced glutathione. Partial separation of two enzyme systems has been achieved; moreover, iodoacetate inhibits the latter without affecting the former. Reduction of insulin appears to proceed independently of any proteolytic action.

The second possible mode of enzymic attack is by proteolysis. It is known that the splitting of only a few peptide bonds in insulin will severely affect biological activity. Nicol (1960) has assayed the biological activity of several purified derivatives of insulin obtained by the action of single proteolytic enzymes. When the *C*-terminal alanine was removed from the *B* chain of insulin by carboxypeptidase the product possessed full activity. However, if the same enzyme was allowed to act for a longer period, biological activity was reduced, as some of the *C*-terminal asparagine was removed from the *A* chain. Trypsin splits from the *C*-terminal part of

TABLE I. Some Properties of the Insulin-Metabolizing and Glucagon-Metabolizing Systems in Liver

Factor	Hormone					
	Insulin (mol. wt. 6,000)			Glucagon (mol. wt. 3,500)		
	Effect on enzyme activity	Method of study	Reference	Effect on enzyme activity	Method of study	Reference
pH	Optimum: 6.5–9.5 7.6–8.0	 B R	 a b	Optimum: 7.0–7.6	 P, B R	 m n
Substrate concentration	*v* max.: 18 μg./min./g. liver K_m: 1.6×10^{-7} M 1.7×10^{-6} M	 R R R	 c c b			
Subcellular fractionation	Activity in supernatant fraction	R B P	j l g	Activity in supernatant fraction	R B P	n p m
Temperature (60° C. for 3–5 min.)	Activity reduced to 30% of original value	R	b	All activity lost	B	p
Ageing (2–3 days at 5° C.)	Activity reduced to 30% of original value	R	e	No loss	B R	p e
Dialysis	Variable: Activity lost No effect	 B R R P	 a e f g	Variable: Partial loss No effect	 P B R	 m p e, n
Addition of citrate	Activity increased to 160% of original value	R	e	No effect	R	e
Addition of glutathione (reduced)	Activity increased	R	d			
Addition of *p*-chloromercuribenzoate, or of Cu^{2+}, or of Zn^{2+}	Inhibition	B R	a b, h	Inhibition	B R	p n
Addition of proteins	Inhibition by: Native insulin Casein Corticotropin Glucagon Somatotropin	R	h, j, k	Inhibition by: Insulin Casein Corticotropin A and B chains of insulin Somatotropin	B P	p, q, r, s m

B: Bio-assay R: Degradation of [^{131}I]iodoinsulin or of [^{131}I]iodoglucagon P: Proteolysis

References: a. Mirsky & Broh-Kahn (1949); b. Mirsky, Perisutti & Dixon (1955); c. Yalow & Berson (1957); d. Williams, Martin, Henley & Swanson (1959); e. Mirsky & Perisutti (1957); f. Narahara, Tomizawa & Williams (1956); g. Vaughan (1954); h. Tomizawa, Nutley, Narahara & Williams (1955); j. Narahara, Tomizawa, Miller & Williams (1955); k. Tomizawa & Williams (1955); l. Mirsky (1953); m. Kenny (unpublished); n. Narahara & Williams (1957); p. Kenny (1956); q. Vuylsteke & de Duve (1955a); r. Vuylsteke & de Duve (1955b); s. Tyberghein, Tomizawa & Williams (1956)

the *B* chain eight amino-acid residues comprising alanine and a heptapeptide. The remaining fragment possesses only 10–28% of the original activity, and the heptapeptide is completely inactive. Thus removal of some of the *C*-terminal residues of insulin may have a marked effect on biological activity. On the other hand, Smith, Hill & Borman (1958) have removed a number of *N*-terminal residues by the action of leucine aminopeptidase without complete inactivation of the molecule.

There is considerable evidence that the inactivation of insulin in liver is associated with its proteolysis. As inactivation or degradation proceeds there is a parallel rise in non-protein nitrogen or ninhydrin colour in the TCA supernatant (Vaughan, 1954; Tomizawa, Nutley, Narahara & Williams, 1955). In addition, autoradiography of the incubation mixture containing [^{131}I]iodoinsulin indicates that the products include small fragments of the original peptide (Mirsky *et al.* 1955; Berson, Yalow & Volk, 1957).

Kenny (1958) has studied the proteolysis at pH 7.4 of fractions *A* and *B* from oxidized insulin (Sanger, 1949) by a dilute supernatant fraction of a rat-liver homogenate. Proteolysis was assessed in a semi-quantitative manner by the intensity of the ninhydrin-stained spots after paper chromatography of the reaction mixture. Under these experimental conditions no significant proteolysis of the intact insulin molecule was observed unless incubation was prolonged (17 hours), or the concentration of the enzyme preparation was increased several-fold. In spite of the stability of the intact insulin molecule, the two oxidized chains were readily attacked, fraction *B* more extensively than fraction *A*. Complete inhibition was obtained with *p*-chloromercuribenzoate.

In further experiments the products obtained by incubation of fraction *A* with the liver preparation were fractionated on filter-paper by a two-dimensional procedure: ionophoresis followed by chromatography with butanol–acetic acid. A number of fractions were visualized after partial staining with 0.01% ninhydrin. They were excised, eluted with 6 N-hydrochloric acid and hydrolysed. The amino-acid composition was determined by further paper chromatography. An enzyme blank, containing no added substrate, was treated in the same way. All the fractions thus identified and attributed to the proteolysis of the oxidized fraction *A* proved to be single amino acids. No peptides were found. The amino acids were: glycine, isoleucine, valine, glutamic acid and glutamine. In addition, there were traces of cysteic acid, alanine and serine. The *N*-terminal sequence of fraction *A* from oxidized ox insulin is as follows: Gly.Ileu.Val.Glu.-$GluNH_2$.$(CySO_3H)_2$.Ala.Ser.Val. (Ryle, Sanger, Smith & Kitai, 1955). The results of this experiment were therefore consistent with the action of an aminopeptidase which hydrolysed, sequentially, single residues from the *N*-terminus of the peptide. The cysteic acid residues may have limited the rate of progress beyond that point in the chain. This conclusion is supported by the observation that acetylation of fraction *A* caused a reduction in proteolysis by the liver enzyme preparation (Kenny, unpublished).

Similar experiments in which fraction *B* of oxidized insulin and glucagon (see section 2) were substrates also showed that free amino acids and not peptides were the products of the liver enzyme system. These findings support the view that an exopeptidase is present in the enzyme system, but a large number of amino acids were identified and a clear choice between an aminopeptidase and a carboxypeptidase could not be made.

Until suitable experiments have been performed with pure enzyme preparations, evidence of the specificity of the insulin-metabolizing system is necessarily of an indirect type. Certainly some degree of enzyme specificity has been established, even with crude preparations. Thus some proteins are not attacked. No hydrolysis of α-lactalbumin or human serum albumin was found (Tomizawa & Williams, 1955) and similarly there was no degradation of the ^{131}I-iodinated forms of human serum albumin, chymotrypsin and lysozyme (Mirsky *et al.* 1955). However, certain other proteins are attacked: corticotropin, somatotropin, casein and glucagon undergo hydrolysis (Tomizawa & Williams, 1955) and the ^{131}I-iodinated forms of ribonuclease, prolactin, pepsin, somatotropin, corticotropin, glucagon and casein are degraded by the crude enzyme preparation which attacks insulin and [^{131}I]iodoinsulin (Mirsky *et al.* 1955; Mirsky & Perisutti, 1957). Some differences between the degradation of [^{131}I]iodoinsulin and the other iodinated substrates have been observed. Ageing or dialysis of the enzyme preparation brought about a reduction, and citrate ions an increase, in the degradation of [^{131}I]iodoinsulin without affecting the degradation of the other substrates (Mirsky & Perisutti, 1957).

It is clear that the liver contains three systems which destroy insulin: (i) a heat-stable, non-enzymic, reducing system; (ii) an enzymic reducing system; and (iii) a proteolytic enzyme system. It is also apparent that studies of biological inactivation or of degradation of [^{131}I]iodoinsulin do not distinguish between the reducing and the proteolytic systems, since both systems can cause inactivation and the formation of TCA-soluble radioactivity.

b. Liver Perfusion Studies

Compared with glucagon, insulin is only slowly inactivated when perfused through the liver. A single passage through an isolated bull-frog liver had no measurable effect; eight perfusions of the same solution were necessary before insulin activity completely disappeared (Weisenfeld *et al.* 1957). Similar experiments have been performed by a cyclic perfusion of [^{131}I]iodoinsulin through the isolated rat liver (Mortimore & Tietze, 1959; Mortimore, Tietze & Stetten, 1959). The fall in TCA-precipitable radioactivity in the perfusate was more rapid than the rise in TCA-soluble radioactivity because of "capture" of radioactivity by the liver. This was attributed to a binding of the [^{131}I]iodoinsulin by the liver amounting to 25% of the total added to the medium. Binding could be studied separately from the phenomenon of degradation by lowering the temperature of the perfusion to 0° C. This temperature abolished degradation but left binding unaffected. The binding of [^{131}I]iodoinsulin showed some specificity in that native insulin competed with the labelled form, while alkali-denatured [^{131}I]iodoinsulin was not bound even after prolonged perfusion.

Perfusion experiments have revealed an interesting biological consequence of inhomogeneity in [^{131}I]iodoinsulin. As commonly used, [^{131}I]iodoinsulin contains one atom of iodine per molecule. However, analysis after hydrolysis showed the presence of di-iodotyrosine, the ratio of di-iodotyrosine to monoiodotyrosine being 0.33. This means that some of the insulin molecules were unlabelled, some had one atom and others two or more atoms of iodine. After perfusion of the liver the ratio of monoiodotyrosine to di-iodotyrosine in the dialysed perfusate had risen to 1.0. This

was interpreted as due to the preferential removal (by binding or degradation) in the liver of the more lightly iodinated molecules of insulin. This recent work serves as a reminder that iodoinsulin is a chemical derivative of the native hormone and may be metabolized in a different manner.

[^{131}I]Iodide was identified as the main radioactive product in the perfusate. This could have arisen in two ways: (i) de-iodination of iodoinsulin or of iodine-labelled peptide fragments derived from it and (ii) de-iodination after hydrolysis and the liberation of free iodotyrosines. By means of isotope dilution experiments with [^{127}I]iodotyrosines, it was possible to prove that the free amino acids and not the larger fragments were de-iodinated in the liver.

c. In-Vivo Studies

The distribution and fate of [^{131}I]iodoinsulin has been studied in vivo in animals and man. When trace amounts of [^{131}I]iodoinsulin are injected into rats, the labelled hormone (measured as TCA-precipitable radioactivity) is rapidly concentrated in the kidney and, to a lesser extent, the liver. These two organs account for about 32% of the dose administered. Other tissues contain much lower concentrations of labelled hormone; some, e.g., erythrocytes, contain none (Elgee, Williams & Lee, 1954). Concentration of [^{131}I]iodoinsulin in the kidneys is not associated with any significant excretion of the hormone in the urine (Narahara, Everett, Simmons & Williams, 1958). This confirms an earlier study of the excretion of native insulin in the urine (Mirsky, Podore, Wachman & Broh-Kahn, 1948). Autoradiography of kidney sections suggests that the labelled hormone is filtered through the glomerulus and then reabsorbed in the proximal convoluted tubule (Narahara *et al.* 1958).

The rate of [^{131}I]iodoinsulin degradation in the intact animal has been assessed by the changes in radioactivity in the plasma. The total and the TCA-precipitable radioactivity fall rapidly immediately after administration, then more slowly (Elgee *et al.* 1954). Berson, Yalow, Bauman, Rothschild & Newerly (1956) have shown that a significant part (28%) of the TCA-precipitable radioactivity in plasma may be distinguished from [^{131}I]iodoinsulin by its migration with plasma proteins on paper electrophoresis. This may be an altered form of the hormone, which results from damage to the insulin molecule caused by radiation. It disappears more slowly from plasma than does the labelled hormone. The latter has a biological half-life of 40 minutes in man (Berson *et al.* 1956) and of 24 minutes in the rabbit (Scott, Prout, Weaver & Asper, 1958). The importance of the liver and kidneys in degrading [^{131}I]iodoinsulin is shown by the delayed fall in the plasma level following hepatectomy or nephrectomy (Elgee & Williams, 1954). Berson *et al.* (1957) have identified [^{131}I]iodide and [^{131}I]monoiodotyrosine among the products in plasma of the degradation of [^{131}I]iodoinsulin. Administration of native insulin at the same time as the labelled hormone delays the degradation of the latter (Berson *et al.* 1957; Wick & Drury, 1958). It has also been shown that the fall in plasma [^{131}I]iodoinsulin follows a similar curve to that of plasma insulin determined by bio-assay. These results support the view that [^{131}I]iodoinsulin and native insulin are metabolized by the same system in the whole animal.

Human diabetes per se does not affect the rate of disappearance of [^{131}I]iodoinsulin from plasma. Previous insulin therapy, however, has the effect of retarding the fall in plasma [^{131}I]iodoinsulin (Berson *et al.* 1956; Welsh, Henley, Williams & Cox, 1956). This phenomenon is explained by the presence in the plasma of insulin-treated diabetics of an antibody to insulin. Insulin bound to the antibody is degraded more slowly than normal in vitro as well as in vivo (Berson *et al.* 1956; Welsh *et al.* 1956; Berson & Yalow, 1957).

2. Metabolism of Glucagon

a. In-Vitro Investigation of the Glucagon-Inactivating and Glucagon-Degrading System

The metabolism of glucagon has been investigated by methods similar to those used in studying insulin. The striking feature of the metabolism of glucagon, compared with that of insulin, is the rapidity with which it is destroyed. A single passage through an isolated frog liver is sufficient to inactivate all the glucagon present in the solution without measurably reducing the insulin content (Goldner, Jauregui & Weisenfeld, 1954). Similarly, extracts of rat liver degrade [^{131}I]iodoglucagon at a rate several times faster than [^{131}I]iodoinsulin (Berson *et al.* 1957).

Liver appears to be the most effective tissue in metabolizing glucagon but other tissues are also active—e.g., kidney, muscle, ileum, spleen, lung, adipose tissue, testis, thymus, and lymph node. Brain and heart muscle appear to be less active; whole blood, plasma and erythrocytes were ineffective (Kenny, 1956; Narahara & Williams, 1957). However, it appears that the plasma enzyme plasmin, if activated by streptokinase, can cause a slow degradation of [^{131}I]iodoglucagon (Mirsky, Perisutti & Davis, 1959); but it seems likely that the contribution of this system is small in relation to enzymes in other tissues. In liver the enzyme systems which inactivate glucagon and degrade [^{131}I]iodoglucagon are localized in the supernatant fraction after removal of the nuclear, mitochondrial and microsomal fractions by centrifugation (Kenny, 1956; Narahara & Williams, 1957).

i. Properties of the Enzyme System in Liver

Table I summarizes some of the properties of the enzyme system in liver.

ii. Inhibitors of the Liver Enzyme

The effect of *p*-chloromercuribenzoate and heavy metal ions shows that the enzyme system is dependent on a thiol enzyme.

Among a number of chelating agents tested, ethylenediaminetetra-acetic acid alone produced partial inhibition of the proteolysis of glucagon in high concentrations. Indole-3-acetic acid and the sulphonylureas, carbutamide and tolbutamide, in high concentrations were also partial inhibitors (Kenny, 1958). These sulphonylureas have previously been shown to inhibit the degradation of [^{131}I]iodoglucagon (Volk *et al.* 1957).

Several proteins have an inhibitory action. Vuylsteke & de Duve (1955a, 1955b) showed that insulin inhibited the inactivation of glucagon by liver slices. The same effect was obtained with cysteine-inactivated insulin, and fractions *A* and *B* from oxidized insulin. The insulin effect has been demonstrated to be a competitive inhibition (Kenny, 1956). Bovine plasma albumin, ribonuclease, and α-lactalbumin were not inhibitors, but other proteins which possessed an inhibitory effect were casein, corticotropin and somatotropin (Kenny, 1956, 1958; Tyberghein, Tomizawa & Williams, 1956). The ability of insulin to protect glucagon

from destruction by liver has led to its use in the assay of glucagon by isolated liver slices. It thereby increases the sensitivity by as much as 100-fold (Vuylsteke & de Duve, 1957).

iii. Nature of the Enzyme

Glucagon is a peptide containing 29 amino-acid residues in a single peptide chain of known sequence (Bromer, Sinn & Behrens, 1957). There are no cystine residues, hence chemical reduction of the molecule is ruled out and the most likely mechanism of inactivation is proteolytic. The evidence for proteolysis is convincing. The enzymic properties of the inactivating system (Kenny, 1956) and the proteolytic (Kenny, 1958 and unpublished work) are essentially similar, and the few differences which were noted were explicable by the different conditions of substrate and enzyme concentration employed in the two types of experiment. Also the appearance of non-protein substances absorbing at 280 mμ correlates well with the degradation of [^{131}I]iodoglucagon (Narahara & Williams, 1957). Finally Berson *et al.* (1957) obtained evidence that the major ^{131}I-labelled product of the degradation of [^{131}I]iodoglucagon was a small but unidentified fragment, possibly a peptide. Kenny (1958) has fractionated the products of the incubation of glucagon with a supernatant fraction of a rat-liver homogenate. All 15 of the amino acids present in the glucagon molecule were identified as free amino acids, or, in the cases of asparagine and glutamine, as the amides. No peptides were found at any stage during incubation. These results suggest the action of an exopeptidase but do not permit a distinction between an aminopeptidase and a carboxypeptidase. However, the results with fraction *A* from oxidized insulin as substrate (see section 1a), and the localization of carboxypeptidase in the mitochondrial rather than the supernatant fraction of the liver (Rademaker & Soons, 1957), favour the choice of an aminopeptidase.

b. In-Vivo Studies

[^{131}I]Iodoglucagon is rapidly degraded in vivo (Berson *et al.* 1957; Cox, Henley, Narahara, Vanarsdel & Williams, 1957). Like [^{131}I]iodoinsulin, [^{131}I]iodoglucagon is concentrated in the kidneys and, to a lesser extent, the liver. Five minutes after intravenous injection the kidneys contain 15% and the liver 11% of the injected [^{131}I]iodoglucagon. Autoradiography of kidney slices suggests that the labelled glucagon is filtered by the glomerulus and reabsorbed in the proximal convoluted tubule (Narahara *et al.* 1958). The role of the liver and kidneys was shown by the reduction in the rate of degradation of the labelled hormone after removal of these organs. Urinary excretion of [^{131}I]iodoglucagon was negligible.

3. Metabolism of some other Peptide Hormones

Although the information concerning other hormones is less detailed than that concerning insulin and glucagon there are several reports which indicate that comparable mechanisms exist. *Vasopressin* and *oxytocin* are rapidly inactivated by extracts of liver, kidney, spleen and small intestine but only slowly destroyed by muscle, uterus and brain. The enzyme which inactivates vasopressin has a pH optimum between 6.2 and 7.0, is inhibited by copper and zinc ions and loses activity on dialysis (Birnie, 1953; Heller, 1957). *Melanocyte-stimulating hormone* is inactivated by liver tissue, though not by boiled or aged liver extracts (Waring & Kirk, 1952). *Adrenocorticotropin* is inactivated by liver, kidney, muscle and adrenal tissues but is unaffected by blood. Boiled tissues also produce some inactivation, but this has been attributed to adsorption or binding on to the denatured protein (Geschwind & Li, 1952). Homogenates prepared from the anterior pituitary inactivate adrenocorticotropin. About half the activity disappears in 15 minutes. The pH optimum is between 7 and 9. Pitressin and glucagon added to the homogenate inhibited the destruction of adrenocorticotropin (Barrett & Sayers, 1958). *Thyrotropin* activity in plasma diminishes following intravenous administration to rats. Very little was recovered in the urine but about 20% of the activity was recovered from the carcass at the end of one hour. Prolonged incubation of thyrotropin with liver slices or thyroid tissue did not lead to inactivation, though there was some loss on perfusion through isolated liver (Levey & Solomon, 1957). There is still some doubt as to the site of inactivation of thyrotropin, though an earlier report suggested that lymphoid tissue might be involved (Rawson, Sterne & Aub, 1942).

4. Conclusions and Summary

The evidence, which has been discussed and which was obtained by a variety of experimental methods, supports the view that the peptide hormones are inactivated in the tissues and that this process is important in limiting the magnitude and duration of hormone action. The peptide hormones appear to be inactivated by degradation rather than by synthetic processes. This is in contrast to the inactivation of the steroid hormones in which conjugation mechanisms play an important part. Insulin is degraded by two known processes—reduction of S—S bonds and proteolysis; of these two it is probable that reduction is the primary event. Glucagon, in common with other peptide hormones lacking cystine residues, is inactivated by proteolytic action only. Because of the similarity in many properties of the insulin-degrading and glucagon-degrading systems it is reasonable to assume that a proteolytic enzyme is common to both systems. Thus the two systems, in liver, have similar pH optima and a similar intracellular distribution. Both are thiol enzymes. Both are inhibited by sulphonylureas, indoleacetic acid, corticotropin and casein. Lastly insulin degradation is inhibited by glucagon, and the proteolysis of glucagon is inhibited by insulin. The main differences in properties of the two systems—the effects of dialysis, ageing and citrate ion—may be referable to the reductive component of the insulin-degrading system.

Evidence has been discussed which suggests that the proteolytic enzyme is an exopeptidase. In any case the presence of an active endopeptidase in contact with cytoplasmic proteins would be an unlikely event. Cathepsin A is known to be localized in the lysosome fraction of the cell, and enzyme activity appears only on rupture of the lysosome membrane (Gianetto & de Duve, 1955). Although the lysosome fraction hydrolyses insulin and glucagon, the enzyme has a much lower pH optimum than the supernatant enzyme and, moreover, is only weakly active when diluted to the same volume as the supernatant fraction (Kenny, unpublished). From the results with fraction *A* of oxidized insulin as substrate, the enzyme in the supernatant fraction appeared to be an aminopeptidase. However, it does not

seem possible to identify the enzyme with other known aminopeptidases. Thus leucine aminopeptidase is widely distributed in tissues and produces extensive hydrolysis of glucagon (Hill & Smith, 1959) and of fractions *A* and *B* from oxidized insulin (Hill & Smith, 1957). But it differs from the enzyme under discussion in having a higher pH optimum, in requiring activation by manganese or magnesium ions, and in being strongly inhibited by citrate but not by heavy metals. Further work with a purified enzyme will be necessary before many of the present uncertainties are resolved.

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INSULIN IN BLOOD

P. J. RANDLE M.A. Ph.D. M.B. B.Chir.

K. W. TAYLOR M.A. Ph.D. M.B. B.Chir.*

Department of Biochemistry
University of Cambridge

Addenbrooke's Hospital, Cambridge

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The detection and estimation of protein hormones in blood present problems of exceptional complexity. Not only is the concentration of a hormone such as insulin in blood very low (*ca* 10^{-8} M), but the biological methods of assay which have been applied to its detection and estimation are neither highly specific nor very precise. The development of methods of separating insulin from interfering substances in plasma before assay is one way of circumventing the problem of specificity, but such a separation has not so far been effected with certainty. Moreover, doubt exists as to the chemical structure and form of insulin in blood, and knowledge of the manner in which it is associated with other serum proteins may be important not only in relation to estimation of the hormone in blood but also in understanding the mechanism of its action.

1. Detection of Insulin in Blood

That insulin exists in blood may at first sight appear self-evident. However, it should be emphasized that circulating insulin has not yet been isolated, nor its amino-acid sequence determined, and until this has been done it would be unwise to assume that insulin in blood necessarily has a chemical structure identical with that of pancreatic insulin. Thus it is already well established that minor changes in the structure of insulin, including species differences in structure, do not necessarily lead to alterations in biological activity (for review see Nichol (1960)). With this proviso, the term insulin is used in what follows to refer to a substance in blood which has many of the physical, chemical and biological properties of pancreatic insulin.

Most methods for the identification of insulin in blood have depended upon known biological effects of the hormone. Thus plasma—or protein fractions of plasma from normal animals, like insulin—lowers the blood-sugar level of intact mice (Beigelman, 1959), hypophysectomized and adrenalectomized rats made diabetic by alloxan (ADHA rats) (Bornstein, 1950), hypophysectomized rats with diabetes induced by alloxan (ADH rats) (Beigelman & Antoniades, 1958), ADH rats from which the adrenal medullae had been removed (AdADH rats) (Hagedorn, 1954), and alloxan-diabetic adrenalectomized mice (ADA mice) (Baird & Bornstein, 1959); increases uptake of glucose, deposition of glycogen and incorporation of [^{14}C]glycine into protein in isolated rat diaphragm in vitro (Groen, Kamminga, Willebrands & Blickman, 1952; Randle, 1954a; Vallance-Owen & Hurlock, 1954; Manchester, Randle & Young, 1959); increases uptake of glucose and $^{14}CO_2$ production from [^{14}C]glucose by rat epididymal fat pad in vitro (Beigelman, 1958; Martin, Renold & Dagenais, 1958); and displaces ^{131}I-labelled insulin from the complex it forms with antiserum to ox insulin produced in guinea-pigs (insulin antiserum) (Yalow & Berson, 1959).

One of the major difficulties with biological methods of detecting insulin in plasma has been the uncertainty as to whether insulin-like effects of plasma are indeed due to insulin. This uncertainty has applied principally to in-vitro methods and there can now be little doubt that insulin-like effects of plasma on uptake of glucose by diaphragm are indeed due principally, if not wholly, to insulin in plasma. Thus, whereas plasma from normal animals increases uptake of glucose by diaphragm, no such effect is seen with plasma from depancreatized, depancreatized-hypophysectomized or depancreatized-adrenalectomized animals unless they have been treated with insulin (Groen *et al.* 1952; Vallance-Owen & Lukens, 1957). Moreover, after treatment with cysteine or alkali (which inactivate insulin), human plasma or acid-ethanol extracts of human plasma no longer stimulate uptake of glucose by diaphragm (Groen *et al.* 1952; Randle, 1954a; Baird & Bornstein, 1957).

Perhaps the most satisfactory evidence for the presence of insulin in plasma has accrued from studies with antiserum to ox insulin prepared in guinea-pigs by methods analogous to that first described by Moloney & Coval (1955). Thus, effects of plasma or protein fractions of plasma from normal oxen or from diabetics treated with ox insulin on uptake of glucose by diaphragm may be completely abolished by addition of insulin antiserum (Randle & Taylor, 1958b; Taylor & Randle, 1959; Wright, 1959). Moreover, effects of rat plasma or acid-ethanol extracts of rat plasma on incorporation of [^{14}C]glycine into diaphragm protein may also be abolished by addition of insulin antiserum (Manchester & Young, 1959). Furthermore, Yalow & Berson (1959) find that human plasma, like human pancreatic insulin, will displace radioactivity from the complex formed between ^{131}I-labelled insulin from ox pancreas and an antiserum to ox insulin. It would seem reasonable to conclude from these observations that plasma from normal animals does contain insulin and that insulin-like effects of plasma on the metabolism of isolated diaphragm are due to insulin in plasma. In view of these findings it would appear to be reasonable to infer also that insulin-like effects of plasma on the metabolism of rat epididymal fat pad or on the blood-sugar level of animal preparations are due to insulin; though it should be emphasized that experimental evidence for such a conclusion is not available.

* Research Fellow in Medicine at Harvard Medical School, USA, 1960.

2. Estimation of Insulin in Blood

a. Micromethods of Insulin Assay

A number of micromethods based on hypoglycaemic effects of insulin in animal preparations or on effects of the hormone on the metabolism of isolated rat diaphragm or rat epididymal fat pad in vitro are now available. Some details of the methods that are both sufficiently sensitive and readily applied to the detection and assay of insulin in blood have been summarized in Table I. A linear relationship between some function of insulin dosage and the response to insulin has been demonstrated for each of these methods of assay. In general, in-vitro methods are much simpler to perform and are as precise and possibly as specific as in-vivo methods, and are to be preferred for these reasons. It is too early yet to draw any firm conclusions about the relative merits of the different in-vitro methods of insulin assay. It may be pointed out, however, that the method based on [^{14}C]glycine incorporation into diaphragm protein is more tedious than the method based on uptake of glucose by diaphragm and neither more precise nor more specific than this. Furthermore, it should be emphasized that, whereas a great deal is now known about the specificity of the method based on uptake of glucose by diaphragm, very little is known at the present time about the specificity of other in-vitro methods.

An immunochemical method of insulin assay has recently been described by Yalow & Berson (1959). It is based upon the fact that insulin-antibody complex may be separated from free insulin by chromatoelectrophoresis. With ^{131}I-labelled insulin of high specific activity (50–100 mc/mg.), conditions may be arranged in which most of the radioactivity is bound by antibody. Addition of unlabelled insulin under these conditions will displace some of the labelled insulin from the complex formed with antibody, and the extent of this displacement (as measured by the change in the ratio of free to bound insulin) is proportional to the amount of unlabelled insulin added. The method appears to be extremely sensitive and rather more precise than other methods and clearly shows great promise.

b. Specificity of Micromethods of Insulin Assay

Although insulin-like effects of plasma or protein fractions of plasma in micromethods of insulin assay appear to be attributable to the hormone, there is now a great deal of evidence that other factors interfere with the estimation of insulin in plasma by these methods. Although most of the evidence has accrued from experiments with isolated rat diaphragm, it would appear likely that these factors may also interfere in other biological methods of assay. The interfering substances include (i) factors in human and rat plasma which are related to pituitary growth hormone and which depress uptake of glucose by diaphragm (i.e., which have an effect opposite to that of insulin) (Bornstein, 1953; Bornstein & Park, 1953; Krahl, Tidball & Bregman, 1959; Taylor, Vargas & Randle, 1960; Vargas, Taylor & Randle, 1960); (ii) factors in human and cat plasma which may be related to growth hormone and which, although without effect on uptake of glucose by diaphragm, nevertheless prevent effects of insulin on glucose uptake (Vallance-Owen & Lukens, 1957; Vallance-Owen, Dennes & Campbell, 1958); and (iii) factors in human plasma which potentiate effects of insulin on uptake of glucose by diaphragm by interfering with the adsorption of insulin to glass and by inhibiting inactivation of insulin by the tissue (Narahara & Williams, 1958; Piazza, Goodner & Freinkel, 1959; Taylor & Randle, 1959). Another factor to be borne in mind with samples of serum from patients or animals treated with heterologous insulin is insulin antibody, which can prevent effects of insulin on uptake of glucose by diaphragm (Berson, Yalow, Bauman, Rothschild & Newerly, 1956; Randle, 1958). It would appear from these observations that the insulin activity of plasma in the diaphragm assay (i.e., the extent to which it stimulates glucose uptake) will depend upon the balance between effects of insulin on the one hand and of factors which diminish or potentiate its effects on the other (Randle, 1954a).

The specificity of other micromethods of insulin assay is much less clearly defined. The uptake of glucose by rat epididymal fat pad is increased by growth hormone, prolactin and corticotropin as well as by insulin (Winegrad, Shaw, Lukens, Stadie & Renold, 1959), though it is not known whether they are present in plasma in sufficient quantity to interfere with the assay of insulin. It is perhaps interesting to note that these hormones do not increase uptake of glucose by diaphragm (Randle & Young, 1957). Potentiating effects of other serum proteins on insulin action may also interfere in the "fat pad assay", though less seriously than in the "diaphragm assay" (Piazza *et al.* 1959). The effects of various factors in plasma which can diminish the action of insulin on diaphragm, in in-vivo methods of assay or the fat pad assay, are not known.

TABLE I. Micromethods of Insulin Assay

	Method of assay	Sensitivity (micro-units/ml.)	Index of precision (λ)	Reference
In vivo	Blood-sugar response of alloxan-diabetic adrenalectomized mouse	1,000	—	Baird & Bornstein (1959)
In vitro	Rat diaphragm:			
	a. Glucose uptake	10–100	0.2–0.4	Groen, Kamminga, Willebrands & Blickman (1952); Vallance-Owen & Hurlock (1954); Randle (1954a, 1956); Wright (1957)
	b. [^{14}C]Glycine incorporation into protein	50	0.4–0.5	Manchester, Randle & Young (1959)
	Rat epididymal fat pad:			
	a. Glucose uptake	10	—	Beigelman (1958)
	b. $^{14}CO_2$ production from [^{14}C]glucose	10	0.31	Martin, Renold & Dagenais (1958)
	c. Net gas output	10–100	—	Ball, Martin & Cooper (1959)
Immunochemical		1.25–5	—	Yalow & Berson (1959)

Nothing is known at present about the specificity of immunochemical methods of insulin assay, though it appears unlikely that the factors which interfere with biological methods of assay will interfere with immunochemical methods. It should be borne in mind, however, that much of the insulin in serum may be combined with other serum proteins and that the kinetics of the reaction between serum insulin and antibody and pancreatic insulin and antibody may be different. If this is so, then it will be necessary to extract insulin from serum before assay by immunochemical methods.

c. Extraction of Insulin from Plasma

It has been emphasized in the foregoing section that plasma contains factors which interfere with the assay of insulin by biological methods and which may interfere with immunochemical methods of assay. Some form of extraction procedure which separates insulin from interfering substances is thus necessary before the hormone can be assayed satisfactorily. Attempts to extract insulin from plasma have involved organic solvents at low pH and ion-exchange resins.

Acid ethanol has long been used to extract insulin from pancreas, and Baird & Bornstein (1957) have described a procedure for the extraction of insulin from plasma which employs a mixture of ethanol, *n*-butanol and toluene acidified with HCl. When ^{35}S-labelled insulin was added to plasma, approximately 80–90% of the radioactivity was extracted by this method. Insulin could be detected in extracts prepared from normal human plasma (Baird & Bornstein, 1957, 1959), and the proportion of insulin to total protein was increased about fortyfold by this procedure. It would be unwise, however, to assume that acid-ethanol extraction necessarily separates insulin from interfering substances in plasma. Acid-ethanol extracts of plasma consist largely of albumin (unpublished observations) and it has been shown that albumin prepared in this way may contain an insulin antagonist (Vallance-Owen *et al.* 1958).

When plasma is equilibrated with the ion-exchange resins IRC 50 or Dowex 50, plasma insulin is retained by the resin (Beigelman, Antoniades, Goets, Renold, Oncley & Thorn, 1956; Antoniades, Beigelman, Pennell, Thorn & Oncley, 1958). The insulin may be recovered in 40–80% yield by elution with 0.3 M citric acid at pH 3 for 72 hr., and the ratio of insulin to total protein increased by 1.5×10^6 (Antoniades, 1958; Antoniades *et al.* 1958). This type of approach to the extraction of insulin from plasma clearly merits further study.

3. Insulin Levels in Plasma or Serum

a. In Normal Animals

The insulin activity of normal human plasma has been assayed by many investigators and by a number of different methods. The results of these experiments are summarized in Table II. Interpretation of these results in terms of the insulin content of plasma is difficult, partly because of the lack of specificity of many of these methods of assay, and partly because in most instances the values have not been derived from satisfactory multi-dose assays (i.e., assays involving more than one dose of standard and unknown). In general terms the values in Table II fall into two main groups: for fasting plasma, 10–350 micro-units/ml. and 100–4,600 micro-units/ml. respectively; and for plasma collected after glucose, 100–800 micro-units/ml. and 1,000–20,000 micro-units/ml. respectively (1 micro-unit = 10^{-6} international unit). The lower values in each case have been obtained from assays of untreated and undiluted plasma by in-vivo or in-vitro methods of biological assay or by an immunochemical method; and the higher values from assays of diluted plasma by in-vitro methods or from assays of extracts of plasma by in-vivo or in-vitro methods. In the "diaphragm assay", interference by factors which oppose the action of insulin may be more marked in assays with undiluted plasma than in assays with diluted plasma (cf. Randle, 1957; Anderson & Levine, 1958; Vallance-Owen *et al.* 1958). In view of this finding, it would appear reasonable to conclude that values obtained with diluted plasma may approximate most closely to the true insulin content, and

TABLE II. Insulin Activity of Normal Human Plasma

	Method of assay	Plasma insulin activity (micro-units/ml.)	Comment	Reference
In vivo	ADHA rat: blood glucose	100	Fasting	
		350	After glucose (2.5 hr.)	Bornstein (1950)
	ADA mouse: blood glucose	800–1,150	Acid-ethanol extract. Fasting	Baird & Bornstein (1959)
		1,700–3,700	Acid-ethanol extract. After food	
In vitro	Rat diaphragm:			
	a. Glucose uptake	10–140	Undiluted plasma. Fasting	Vallance-Owen & Hurlock (1954)
		100–800	One hour after oral glucose	Wright (1957); Seltzer & Smith (1959)
		100–4,600	Diluted plasma. Fasting	Willebrands & Groen (1956); Willebrands, van der Geld & Groen (1958)
		1,000–2,000	Acid-ethanol extract. Fasting	Baird & Bornstein (1957)
		1,000–20,000	Diluted plasma; 2.5 hr. after glucose	Randle (1954a, 1957)
	b. [^{14}C]Glycine incorporation into protein	2,000–10,000	Diluted plasma; 2.5 hr. after glucose	Manchester, Randle & Young (1959)
	Rat epididymal fat pad:			
	a. Glucose uptake	250–1,000	Undiluted or diluted plasma	Beigelman & Antoniades (1958)
	b. $^{14}CO_2$ production from [^{14}C]glucose	50–350	Undiluted plasma. Fasting	Martin, Renold & Dagenais (1958)
Immunochemical		64–98	Fasting	Yalow & Berson (1959)
		236–302	After glucose (1 hr.)	

results obtained from assays of acid-ethanol extracts of plasma are in keeping with this view. Although much less is known concerning the specificity of the fat pad assay or in-vivo methods of assay or immunochemical methods, it would appear that values obtained by these methods are likely to be too low rather than too high, for reasons given elsewhere. We would conclude that the concentration of insulin in plasma may be as high as 1,000 micro-units/ml. in fasting subjects and of the order of 3,000–5,000 micro-units/ml. after glucose (*ca* 10^{-8} M), though it should be emphasized that the experimental evidence is far from conclusive. We would also conclude that the plasma level of insulin is increased after oral administration of glucose in normal people.

b. In Diabetic Patients

Studies of plasma levels of insulin activity in diabetic patients have been largely directed towards an assessment of the part played by insulin deficiency in the two broad clinical types of diabetic. The earliest studies were carried out by Bornstein & Trewhella (1951) and Bornstein & Lawrence (1951a, 1951b), who estimated plasma insulin activity by the blood-sugar response of ADHA rats. They have concluded that the plasma level of insulin activity is very low before treatment with insulin in diabetics, usually young, who present with weight loss and ketosis. After treatment with insulin in this group of diabetics, the plasma level of insulin activity approximated to the normal level. In diabetics before treatment who were generally middle-aged and obese and without ketosis and who were subsequently well controlled with diet alone, the plasma level of insulin activity approximated to the normal level. Essentially similar findings were reported by Baird & Bornstein (1957, 1959) who assayed insulin activity in acid-ethanol extracts of plasma by the glucose uptake of diaphragm or the blood-sugar response of ADA mice.

The level of plasma insulin activity in diabetic patients fasting and after glucose has been assayed by the diaphragm method by Vallance-Owen, Hurlock & Please (1955). They find that the plasma level of insulin activity is perhaps above normal in the fasting state in diabetics who do not require insulin treatment, though perhaps below normal after glucose (cf. Seltzer & Smith, 1959). In diabetics requiring insulin, the plasma level of insulin activity was very low if control was poor, though normal when control was good, but this difference appears to be due to the activity of an insulin antagonist in the plasma of poorly controlled diabetics rather than to any marked change in the plasma level of insulin. Thus, when plasma is assayed after dilution (which diminishes the activity of an insulin antagonist—Vallance-Owen *et al.* 1958), insulin can readily be detected in the plasma of insulin-treated diabetics who are poorly controlled (Vallance-Owen, personal communication; Randle, unpublished observations; Groen *et al.* 1952; Willebrands & Groen, 1956).

Although the methods of assay employed in these studies lack specificity, the results obtained do support the view that insulin deficiency is responsible for the development of diabetes in diabetics requiring insulin and that factors other than insulin deficiency are responsible in diabetics not requiring insulin. A further important point, which now appears well established, is that a small number of insulin-requiring diabetics may have a substantial amount of insulin in the plasma at the time of onset of diabetes even when diabetic ketosis is present (Baird & Bornstein, 1957, 1959; Taylor, 1960). This finding perhaps offers hope of remission to a small proportion of those with severe diabetes (cf. Taylor, 1960).

c. Effects of the Pituitary

After hypophysectomy in the rat, or in cases of panhypopituitarism in man, plasma insulin activity may be markedly reduced (Randle, 1954c; Randle & Young, 1956). This change may be due principally to deficiency of growth hormone in these conditions, as treatment with growth hormone may prevent a fall in plasma insulin activity after hypophysectomy in the rat (Randle & Young, 1956). In keeping with this view, it has been found that plasma insulin activity is increased in active cases of acromegaly (Randle, 1954b; Candela, 1956; Willebrands & Groen, 1956; Wright, 1957) and in the cat following treatment with growth hormone (Randle & Young, 1956).

d. Effects of Hypoglycaemic Sulphonamide Derivatives

Loubatières (1946), on the basis of cross-circulation experiments, first suggested that hypoglycaemic sulphonamides might lower the blood-glucose level by stimulating insulin secretion. More recently Pfeiffer, Pfeiffer, Ditschuneit & Ahn (1959) and Vallance-Owen, Joplin & Russell Fraser (1959) have obtained evidence that the plasma level of insulin is increased in normal people and in diabetic patients who show a hypoglycaemic response to the administration of tolbutamide.

4. Chemical Activity of Insulin in Serum

a. In Normal People

When proteins are separated from normal human plasma by fractional precipitation, insulin may be detected in fractions containing β-globulins (Beigelman *et al.* 1956; Taylor, Vargas & Randle, 1959; Vargas *et al.* 1960) and in a fraction containing albumin and α_1-globulins, but not in γ-globulins (Taylor *et al.* 1959; Vargas *et al.* 1960). Similarly, when plasma protein fractions are prepared by zone electrophoresis on columns of treated cellulose (Randle & Taylor, 1958a; Taylor & Randle, 1959) or starch (Bolinger, van der Geld & Willebrands, 1959) or by curtain electrophoresis (Beigelman, 1958, 1959; Beigelman & Onoprienko, 1959), insulin may also be detected in β-globulins and (with columns of treated cellulose which have been equilibrated with normal human plasma) in fractions containing albumin and α_1-globulin. Some insulin was also detected in γ-globulins, but this may be due to contamination of γ-globulins with β-globulins containing insulin and not to the presence of insulin in γ-globulins. The significance of these findings lies in the fact that, whereas much of the insulin in serum migrates on electrophoresis with β-globulins, insulin from ox pancreas (whether unlabelled or labelled with ^{131}I) migrates between albumin and α_1-globulins (Berson *et al.* 1956; Kalant, Gomberg & Schucher, 1958; Randle & Taylor, 1958a; Skom & Talmage, 1958). This has led to the suggestion that normal human serum contains both free insulin (migrating in the zone of albumin and α_1-globulins) and a bound form of the hormone, i.e., combined with another serum protein (migrating in the β-globulin zone) (Randle & Taylor, 1958b; Taylor & Randle, 1959). It remains to be established whether the bound form of insulin in human plasma is due to an association between insulin and other serum proteins of a non-specific

kind, or whether it represents a specific transport form of the hormone in plasma. In this connexion, in preliminary studies plasma from human portal vein blood (from a case of islet cell adenoma and a case of myelosclerosis) has yielded albumin and α_1-globulin fractions containing substantial quantities of insulin and β-globulin fractions in which no insulin could be detected. These results would perhaps support the view that the association of insulin with β-globulins may be a specific one, but further studies are clearly required to establish this point.

Further evidence for altered chemical activity of insulin in normal human plasma has accrued from studies by Antoniades (1958) and Antoniades *et al.* (1958) who find that insulin in human plasma is retained by the ion-exchange resins IRC 50 and Dowex 50 under conditions where insulin from ox pancreas is not retained by the resin.

b. In Insulin-Treated Diabetics

When ^{131}I-labelled ox insulin is subjected to zone electrophoresis in the presence of normal human serum, radioactivity migrates in the zone of albumin and α_1-globulins (see foregoing section). On the other hand, when electrophoresis is carried out in the presence of serum from normal people or from diabetic patients treated for several months with ox insulin, much of the radioactivity migrates between β- and γ-globulins (Berson *et al.* 1956; Kalant *et al.* 1958). This finding has led to the conclusion that serum from patients treated with heterologous insulin contains an insulin-binding antibody (Berson *et al.* 1956), and it is in keeping with observations concerning the appearance of antibodies to insulin in guinea-pigs or horses treated with heterologous insulins (Moloney & Coval, 1955; Moloney & Aprile, 1959; Yalow & Berson, 1959).

Although ^{131}I-labelled ox insulin behaves otherwise on electrophoresis in serum from normal people than it does in serum from diabetic patients treated with ox insulin, it is interesting to note that the distribution of endogenous insulin in protein fractions of serum is very similar in both instances (Randle & Taylor, 1958b; Taylor & Randle, 1959). The possibility that so-called insulin-binding antibody may be a variant of a normal transport protein for insulin in blood is one which merits further study.

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INSULIN ANTAGONISTS

J. VALLANCE-OWEN M.A. M.D. M.R.C.P.

Department of Medicine
King's College, University of Durham

1 Insulin antagonists in tissues
2 Insulin antagonists in plasma and serum
a Plasma and serum from experimental animals
b Plasma and serum from normal and diabetic human subjects
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An insulin antagonist may be defined as any substance or fraction which can either modify or combine with insulin to render it inactive, or counteract its effects in vivo or in vitro. Many individual hormones, notably growth hormone and the adrenal cortical steroids, have been called physiological insulin antagonists, on the basis of their ability to raise the blood-sugar level, produce insulin-resistance or counteract hypoglycaemia induced in vivo by insulin. An extensive review of such antagonists has been recently published (de Bodo & Altszuler, 1958), and these hormones will necessarily be mentioned again in relation to "Endocrine disorders and insulin action" (discussed in paper by Russell Fraser, p. 242 of this symposium). They will be mentioned here only in relation to the several hormone-dependent anti-insulin substances found in plasma and serum.

Again, insulin antibodies can be antagonists. Such antagonists are iatrogenic, occurring only as a result of insulin therapy. This aspect of insulin antagonism will be discussed in the paper by Wright, p. 219 of this Bulletin.

The other antagonistic factors found in plasma or serum may be distinguished from antibodies in that they can be demonstrated without prior administration of exogenous insulin. Although these humoral insulin antagonists are of considerable interest, little is known as yet about their character and even less about their mode of action.

However, one insulin antagonist has been extensively studied. It is an enzyme system found in many body-tissues and is called insulinase; it is responsible for the proteolytic breakdown of insulin.

1. Insulin Antagonists in Tissues

Early observations clearly showed that insulin can be destroyed rapidly in vivo and in vitro (see, for example, Brugsch & Horsters, 1927; Collins & Murlin, 1929), but nothing was known of the mechanism involved in the process.

Since 1949 Mirsky and his collaborators have been particularly active in elucidating this problem. The data that they have accumulated indicate that the liver and, to a lesser extent, other tissues possess a system capable of destroying and inactivating insulin, and this they have termed "insulinase". In their early studies on tissue extracts and homogenates, the hypoglycaemic response of rabbits to intravenous injections of insulin, before and after incubation with such preparations, was used to estimate insulinase activity, the hypoglycaemic response obtained being inversely proportional to this activity (Broh-Kahn & Mirsky, 1949; Mirsky & Broh-Kahn, 1949). They found that, in the rat, the greatest activity was present in the liver and kidney; some activity was demonstrable in skeletal muscle, while none could be detected in the brain. They also showed that the inactivating system had the properties of an enzyme and was dependent upon the participation of the sulphydryl group.

With the advent of radioactive iodine ^{131}I, it became possible to label insulin with this isotope without detectable loss of biological activity. Moreover, this labelled insulin has the same electrophoretic mobility as native insulin. Thus, the fate of the insulin molecule in vivo and in vitro can be determined reasonably easily; after treatment of tissues with trichloroacetic acid, the release of ^{131}I-labelled fragments into non-protein fractions serves as a reasonably precise measure of the quantity of labelled insulin broken down or degraded over any given period of time.

Most of the detailed studies on insulinase have been carried out with liver preparations. Following ultracentrifugation, the fraction of a liver homogenate which remains after the removal of mitochondria, microsomes and nuclei, degrades ^{131}I-labelled insulin most rapidly (Mirsky, 1953; Vaughan, 1954; Beyer, 1955; Narahara, Tomizawa, Miller & Williams, 1955). The rapid degradation of insulin by these fractions is apparently caused by two factors, one heat-stable, the other heat-labile, the latter being responsible for about 75% of the activity. The heat-stable factor is readily dialysable, withstands boiling and is believed to inactivate insulin by reducing one or more of its disulphide bonds. Although Mirsky & Broh-Kahn (1949) originally gave the name "insulinase" to the whole insulin-inactivating system found in homogenates and extracts of various tissues, it is now reserved for the heat-labile component of this system. This component can be separated from the heat-stable factor and concentrated by various procedures involving ammonium sulphate fractionation (Vaughan, 1954; Tomizawa & Williams, 1955). This principle is found to be most active in destroying insulin between pH 6.5 and pH 9.5 at a temperature of 46° C. There is progressive loss of activity as the temperature falls to zero (Mirsky, 1953; Mirsky, Perisutti & Dixon, 1955). Heating to 80° C. for 10 minutes or oxidation of —SH groups will completely abolish the insulin-degrading activity.

When ^{131}I insulin is incubated with liver homogenates, the supernatant fraction left after protein precipitation with trichloroacetic acid shows comparable increases in both the radioactivity and nitrogen (Mirsky, Perisutti & Dixon, 1954; Tomizawa & Williams, 1955), and the loss of activity can also be related to a rise in non-protein colour produced by ninhydrin (Vaughan, 1954). Taken together, these observations strongly suggest that the insulinase system is a proteolytic enzyme. The activity of this system can be modified in various ways. It can be reduced by fasting and rapidly restored by high carbohydrate diet (Mirsky, Perisutti & Jinks, 1955). Also it is apparently hormone-dependent, or partially so, as the removal of the pituitary or thyroid gland in rats reduces the activity of their liver homogenates very considerably (Elgee & Williams, 1955). This activity is restored to normal in the thyroidectomized animals by injection of thyroxine. However, the absolute specificity of this enzyme

system for insulin, as maintained by Mirsky (see, for example, Mirsky, 1957) and as implied by its name, has been challenged by Tomizawa & Williams (1955). These authors showed that α-corticotropin, casein, glucagon and growth hormone may also be substrates for this heat-labile insulinase system.

During the course of attempts to purify the system responsible for the inactivation of insulin by liver, Mirsky and his collaborators identified an insulinase inhibitor in this organ and other tissues of the rat (Mirsky, Simkin & Broh-Kahn, 1950). This inhibitor is readily dialysable; it is also destroyed by alkali, but resistant to heat and to the proteolytic enzymes, pepsin and trypsin. The activity of this insulinase inhibitor, which appears to be competitive in nature, has been demonstrated in vivo and in vitro (Mirsky *et al.* 1950; Mirsky & Perisutti, 1955).

Although the specificity of this insulinase system is open to question, there can be little doubt that insulinase is responsible for the relatively short life-span of insulin in vivo. This is a well-known physiological phenomenon, half the circulating insulin being degraded in about 40 minutes in man (Berson, Yalow, Bauman, Rothschild & Newerly, 1956).

The possibility that excessive insulinase inhibitor may be of importance in the aetiology of diabetes mellitus has been postulated by Mirsky (1952, 1957). An increase in insulinase activity has been reported in liver tissue from patients with diabetes when compared with that from non-diabetic controls (Mirsky, 1956). However, insulin appears to be protected from degradation by the liver in the presence of plasma from diabetic patients who have been treated with insulin (Yalow & Berson, 1957). It is even more significant that Berson *et al.* (1956) have failed to show an increased rate of insulin degradation in untreated diabetic patients. At present, then, it remains to be shown that insulinase, and its naturally occurring inhibitor, have any significance in the aetiology of diabetes mellitus.

2. Insulin Antagonists in Plasma and Serum

Some confusion at present surrounds the question of these insulin antagonists. Several apparently different factors have been described in plasma or serum which can antagonize insulin activity or which have an effect opposite to that of the hormone.

Small amounts of insulin accelerate both the utilization of glucose and the synthesis of glycogen by the isolated rat diaphragm (Gemmill, 1939, 1941). Also a quantitative relationship has been shown to exist between the concentration of insulin in the incubation medium and its effects on the glucose metabolism of the diaphragm (Stadie & Zapp, 1947; Krahl & Park, 1948). Most investigators interested in this problem have used variations of this diaphragm technique as their test system for measuring insulin activity and antagonism in plasma or serum (see also paper by Randle & Taylor, p. 209 of this Bulletin).

a. Plasma and Serum from Experimental Animals

i. Rat

The glucose uptake by normal rat diaphragms in various types of sera was studied by Bornstein & Park (1953). They confirmed the findings of Tuerkischer & Wertheimer (1948) that the serum from alloxan-diabetic rats produced significantly less glucose uptake and hence insulin effect than serum from normal rats.

If either hypophysectomy or adrenalectomy was performed on these alloxan-diabetic animals, then their serum gave substantially the same glucose uptake as that from normal rats. When growth hormone and cortisone were injected into hypophysectomized alloxan-diabetic rats, then their serum again gave low glucose uptake values; neither growth hormone nor cortisone would give this effect if injected alone. Also the in-vitro addition of either or both hormones to the serum of hypophysectomized alloxan-diabetic animals did not lower glucose uptake; this indicates the need for body-passage of these hormones to produce this effect. These findings were confirmed by Whitney & Young (1957). From these observations it has been inferred that there is a hormone-dependent insulin antagonist in the serum of alloxan-diabetic rats, which is responsible for the reduced effect of the contained insulin.

However, when insulin in a final concentration of 100 milliunits/ml. was added to the serum from these animals, there was no reduction in the insulin effect. This could be owing to the relatively large amount of insulin added in vitro, but the more probable reason was that the antagonist was fully utilized in inhibiting the insulin already present in the serum, leaving none to handle any additional insulin.

The antagonistic factor in the serum of alloxan-diabetic rats appeared to be associated with the β_1-lipoprotein fraction and was inactivated by freezing or standing in an ice-bath (Bornstein, 1953). The serum of such rats after removal of the lipoprotein was not inhibiting. Normal rat and human serum and that from hypophysectomized rats failed to yield an inhibitory lipoprotein fraction.

In line with the above observations, Randle (1955) noted that low insulin activity of plasma from alloxan-diabetic rats was a feature only of fresh plasma and that plasma that had been repeatedly frozen and thawed possessed normal insulin activity. Also Hendley, Bregman & Krahl (1957) found that the antagonistic activity of sera from diabetic rats towards glucose uptake parallels the lipoprotein content in a general way. However, there was no detailed correlation between inhibitory activity of individual serum samples and their lipoprotein content. Recently, uniformly inhibitory lipoprotein fractions have been prepared, not only from sera from diabetic rats but from normal rat sera also. These fractions have been shown to inhibit the stimulating effect of 0.3 milliunit/ml. of added insulin on glucose uptake (Krahl, Tidball & Bregman, 1959).

It is important to stress that this lipoprotein antagonist in serum modifies the effect of any contained insulin and, when separated as a fraction, can also antagonize insulin added in vitro. All the above observations and comparisons have been carried out with incubation media containing insulin, e.g., sera from normal or alloxan-diabetic rats, 3%, 4% or 6% crude bovine albumin. This crude albumin has a stimulatory effect later shown to be due to contamination of such preparations with insulin (Randle & Taylor, 1958).

Thus, there is no evidence, as yet, that the inhibitor reduces the uptake of the diaphragm below basal uptake, that is, below the amount taken up by the tissue when no insulin is added to the medium.

ii. Cat

The plasma from a cat rendered permanently diabetic by prolonged treatment with growth hormone (metahypophysial diabetes), when frozen and thawed caused significantly greater glucose uptake than fresh plasma from the same animal

(Randle & Young, 1956), suggesting that a lipoprotein type of insulin antagonist might be present in this form of diabetic cat. However, the glucose uptake of normal cat plasma, subjected to the same manoeuvre, was not altered, indicating that an insulin antagonist similar to that found in alloxan-diabetic and normal rats was not present in the untreated animal.

Using the diaphragm technique, insulin antagonism of plasma from normal, depancreatized, depancreatized-hypophysectomized and depancreatized-adrenalectomized animals has been studied with and without various hormonal replacements (Vallance-Owen & Lukens, 1957). Plasma from normal fasting cats showed insulin activity; and, when a known amount of insulin was added to the plasma in vitro, its activity was not diminished. In the plasma of depancreatized cats there was no measurable insulin activity, and this plasma strikingly inhibited the effect of 1 milli-unit/ml. of insulin added—only 15% was recovered.

This antagonism within plasma from depancreatized cats was removed either by hypophysectomy or adrenalectomy. In either depancreatized-hypophysectomized or depancreatized-adrenalectomized cats there was again no plasma insulin activity, but now the activity of insulin added to the plasma was fully recovered—that is, as in the normal animal, there is no demonstrable antagonism in their plasma. Treatment with cortisone or hydrocortisone restored the antagonistic properties to the plasma of depancreatized-adrenalectomized animals but not to that from depancreatized-hypophysectomized preparations. Growth hormone injected into either type of animal also failed to restore the antagonistic properties to their plasma. It was therefore concluded that, as with alloxan-diabetic rats, the insulin antagonism found in the plasma of depancreatized cats was the result of the combined activity of the pituitary gland and the adrenal cortical steroids.

Although it is not yet known where this antagonism resides in the plasma, it was not affected by repeated freezing and thawing, and thus is different from the factor found in alloxan-diabetic rats and is unlikely to be a lipoprotein.

b. Plasma and Serum from Normal and Diabetic Human Subjects

When insulin was added in vitro to the plasma from normal human subjects, there was no detectable insulin antagonism (Vallance-Owen & Hurlock, 1954). This is also true of plasma obtained from obese diabetic patients, who show no tendency to develop ketosis unless their diabetes is complicated by infection and who ordinarily do not require insulin therapy (Vallance-Owen, Hurlock & Please, 1955).

However, it was also found that the plasma from untreated or uncontrolled but non-ketotic diabetic patients, who require insulin treatment, possessed insulin antagonism; it was demonstrated by inhibition of the activity of insulin added in vitro upon the glucose uptake of rat diaphragm (Vallance-Owen *et al.* 1955). If the disease in these patients was controlled with additional insulin so that the blood sugar was present in physiological amounts at the time of the test, then there was no demonstrable antagonism, the activity of additional insulin being fully recovered. These observations suggested that these diabetics require insulin to overcome some antagonist circulating in their plasma.

Subsequent experiments have shown that the antagonism to insulin present in these patients resides in the albumin fraction of the plasma proteins prepared either by a trichloroacetic acid–ethanol method or by ethanol or ether fractionation (Vallance-Owen, Dennes & Campbell, 1958a). These authors also found that a substance antagonistic to insulin was present in the albumin fraction of plasma from normal subjects, though this fraction was less active than that from the plasma of the diabetic patients.

Albumin prepared from the plasma of diabetics requiring insulin and from normal subjects, when passed through a column of partially acetylated cellulose, lost its antagonistic activity to insulin, although it was still electrophoretically identical with albumin. Also it was observed that the plasma albumin fraction from hypophysectomized patients was devoid of insulin antagonism (Vallance-Owen, Dennes & Campbell, 1958b).

Together, both these lines of evidence indicate that the anti-insulin activity of the albumin fraction from normal subjects and diabetic patients is not due to albumin per se but to a substance associated with it. The results of hypophysectomy indicate that the antagonistic activity is related, possibly indirectly, to the pituitary gland. This gland is probably not solely responsible, however, as albumin prepared from the plasma of two totally adrenalectomized patients, in whom cortisone therapy had been discontinued for at least 50 hours, also failed to antagonize insulin (Vallance-Owen, unpublished observations). These findings are similar to those relating to the insulin antagonism in depancreatized cats and in alloxan-diabetic rats, already described.

It is now apparent that the insulin antagonist can be separated from human albumin by methods which may, or may not, involve protein denaturation. The antagonist is rendered dialysable either by heat coagulation of an albumin solution, by suspension of albumin in an organic liquid or by solution of albumin in normal acetic acid or ammonia. The active principle presumably has a low molecular weight and seems unlikely to be a protein, free lipid, fatty acid or steroid from the experimental evidence so far accumulated (Vallance-Owen & Lilley, 1960). This antagonist, like the others so far mentioned, inhibits the stimulating action of insulin on the glucose uptake of the diaphragm. It does not reduce the glucose uptake below that achieved by the diaphragm when no insulin is present in the incubation medium.

In the plasma of some diabetic patients in severe acidosis, another—and possibly distinct—insulin antagonist has been detected (Field & Stetten, 1956a, 1956b). In this study the insulin antagonism was determined, using Stadie's "dipping" technique and measuring the glycogen deposition in the rat diaphragm (Stadie, Haugaard, Marsh & Hills, 1949); this technique is considerably less sensitive than that in which glucose uptake by the diaphragm is determined. Consequently, large amounts of insulin were used to detect antagonism. This antagonism in the plasma of severely ketotic diabetic patients was evanescent, disappearing within a few hours after the commencement of insulin therapy. It was thought to be responsible for the high doses of insulin necessary in many instances for the control of diabetic coma. These and subsequent investigations showed that the antagonist migrated electrophoretically with the α_1-globulin fraction of the plasma proteins; was stable to freezing and to heat (60° C.); was destroyed by chymotrypsin but not by trypsin; and was not due to the low pH of such plasma, or to the physical presence of glucagon, growth hormone or adrenal hormones (Field, Tietze & Stetten, 1957). These workers were unable to suggest the nature of this antagonist but were able to show that it

could not be detected by them in plasma of diabetics unless these were acidotic. Nevertheless, in view of the relatively insensitive assay used, it is possible that this α_1-globulin antagonist is the same as that shown to be associated with the albumin fraction of the plasma from normal subjects and non-ketotic diabetic patients; this antagonist could be detected by the above method only if it was considerably increased in amount, as is likely to occur when acidosis supervenes in the diabetic state. Also most methods of protein fractionation are not sufficiently precise to exclude the possibility of some cross-contamination with closely allied fractions.

Baird & Bornstein (1957) used a mixture of acid butanol, ethanol and toluene to extract insulin from normal and diabetic human plasma. They found that the residue left after this extraction frequently caused a significant reduction in glucose uptake of rat diaphragms below the level of that in controls, i.e., with no insulin in the incubation medium. These workers did not identify the factor responsible. A similar finding was reported by Taylor, Vargas & Randle (1959) using ethanol fractionation techniques on normal human serum. They found that the inhibitory component consisted mainly of albumin, with small quantities of α-globulin and β-globulin. Subsequent column electrophoresis localized the inhibitor in the α_2-globulin and β-globulin fractions (Randle and Taylor, personal communication).

c. Discussion

Although several humoral insulin antagonists have been described, there are points of similarity as well as differences between them; and the differences may be due, in part at least, to imperfections in assay and extraction techniques.

The antagonist in the plasma from alloxan-diabetic and normal rats, depancreatized cats, normal subjects and insulin-requiring diabetic patients is hormone-dependent. In each case the pituitary gland and the adrenal cortical steroids must be present for insulin antagonism to occur. The fact that β-lipoproteins are responsible for the antagonism in the rat, but not in the cat or in man, may be merely a species difference. Also it is as yet by no means certain that the α_1-globulin antagonist found in the blood of severely acidotic diabetics differs from that associated with the albumin fraction in non-ketotic diabetics and normal subjects.

The "antagonist" in the residue from the extraction of insulin by organic solvents from plasma, and that found in the α_2-globulin and the β-globulins, both appear to be different from those discussed in the previous paragraph. They depress the glucose uptake of isolated muscle tissue in the absence of added insulin and may appear to be antagonistic to the action of insulin only because of this action. These two factors may be identical, but as yet little is known about them.

Normal Subjects

Although many questions remain unanswered about insulin antagonists in normal plasma whose existence is ordinarily masked by adequate insulin production, it seems probable that antagonists play an important role in insulin metabolism, and through it in that of carbohydrate, fat and protein.

It is well known that a high concentration of glucose in the blood circulating through the pancreas stimulates insulin secretion by the islets (e.g., Anderson & Long, 1947; Foa, Weinstein & Smith, 1949). However, in normal subjects major fluctuations in blood-sugar concentration do not occur. Thus, although the liver plays an important role in this connexion, it may be that the normoglycaemic state is maintained more by variations in humoral antagonistic activity than by fluctuations in the rate of insulin secretion by the pancreas. For example, an increase in these antagonists would prevent any excess insulin activity after it has produced its desired effect and before it has been removed from the blood. Similarly, a reduction in these factors would allow an increase in insulin effect from the insulin already circulating; this, in many circumstances, may be all that is required to restore metabolic equilibrium. However, on occasions this may be insufficient for the needs of the body, so that more insulin has to be produced by the islets. In other words, the coarse adjustment, taking time, is the output of insulin from the pancreas; whereas the fine adjustment, acting quickly, is mediated by the antagonist which, in turn, is apparently controlled by the pituitary–adrenal system.

The insulin-resistance of diabetic acromegalics and patients with Cushing's syndrome, and the insulin-sensitivity exhibited by patients with Simmonds' or Addison's disease, are entirely in accord with this concept. Also, as the pituitary–adrenal system is essential for life, the appearance of insulin antagonism in the plasma of depancreatized animals would be expected.

Insulin-Requiring Diabetic Patients

When insulin-requiring diabetics are untreated or uncontrolled, any insulin added to their plasma in vitro is antagonized. This observation suggests that these diabetics require exogenous insulin to overcome some antagonist circulating in their plasma. In order to achieve control, sufficient insulin must be given, not only to counteract this antagonism but also to leave sufficient active or free active insulin to carry out its normal function. It is entirely possible, at least initially, that there is some endogenous insulin production in these patients which is completely masked by the antagonistic activity. On the available evidence, it seems highly likely that the insulin antagonism found in these insulin-requiring diabetics is merely an exaggeration of the situation found in normal subjects where the antagonism is completely masked by adequate insulin production from the pancreas (Vallance-Owen, 1959). In both normal and diabetic subjects, one insulin antagonist is associated with the albumin fraction of the plasma proteins and is controlled through the pituitary–adrenal system. In each case the antagonism is not due to albumin itself but to a substance associated with it. In view of the increased antagonistic activity of the diabetic plasma protein, this substance may well be present in larger amounts on diabetic albumin than on normal albumin.

We can infer from this that insulin-requiring diabetics either have from birth, or gradually develop, more insulin antagonism than normal subjects, mediated through over-action of the pituitary–adrenal system, but to a degree, as yet, quite unmeasurable. This slightly increased antagonism to insulin, operating over a prolonged period, finally exhausts the β cells of the pancreas, which for genetic reasons may be less resilient than the corresponding cells in normal subjects, and thus diabetes develops. Indeed it seems possible that the condition could arise solely from over-action of the pituitary–adrenal system, this being the fundamental abnormality; as this activity fluctuates to some extent throughout the day, the "brittleness" or difficulty in controlling many diabetics even under optimum conditions and with full co-operation can be explained. If this hypothesis is correct, then it may be expected that giving adrenal cortical steroids, in sufficient quantity to

suppress endogenous production of these hormones, will stabilize "brittle" diabetics. This has already been shown (Vallance-Owen, 1960). As many authorities now believe that hypophysectomy or bilateral adrenalectomy can arrest the progress of diabetic complications, the above observations have more than theoretical interest.

Further study of humoral insulin antagonists merits urgent attention. It may lead not only to a better understanding of carbohydrate and fat metabolism, and, through it, of the syndrome of diabetes mellitus, but also give information about the cause and subsequent prevention of the serious complications that are so often associated with this condition.

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INSULIN ANTIBODIES

P. H. WRIGHT M.Sc. M.B. Ch.B.

Department of Chemical Pathology
Guy's Hospital Medical School, London

1 Detection of insulin antibodies
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An antibody has been defined as ". . . any substance which makes its appearance in the blood serum and body fluids of an animal, in response to the stimulus provided by the introduction of an antigen into the tissues, and reacts specifically with that antigen in some observable way" (Wilson & Miles, 1946). Since it was first used therapeutically, insulin, which is a protein of low molecular weight (Brown, Sanger & Kitai, 1955), has been considered a potential antigen, and its antibodies have been sought in the serum of animals and men submitted to insulin treatment. At first many of the immunological reactions observed in diabetic patients were attributed to impurities in the insulin preparations then in use (Wasserman, Broh-Kahn & Mirsky, 1940), but later it became obvious that the hormone itself was capable of inducing antibody production.

Two types of reaction in insulin-treated diabetic patients have been attributed to the immunological properties of the hormone. First, localized or, more rarely, generalized allergic skin reactions may render insulin therapy difficult. These are thought to be due to insulin antibodies of the "reagin" type (Davidson & Eddleman, 1950; Loveless & Cann, 1955) and have been avoided by the use of highly purified crystalline insulin preparations (Jorpes, 1950) or controlled by treatment with antihistamines (Davidson & Eddleman, 1950). The second type of antibody is thought to play a major part in the production of insulin-resistance in a small but important group of diabetic patients. The problem of resistance to insulin therapy has been reviewed on several occasions (Glen & Eaton, 1938; Smelo, 1948; Berne & Wallerstein, 1950; Davidson & Eddleman, 1950; Kleeberg, Diengott & Gottfried, 1956), and the parts played by insulin antagonists and antibodies in its production have recently been discussed briefly (Berson & Yalow, 1958). Insulin allergy and resistance to insulin therapy appear to be associated with different antibodies, for both phenomena can appear in the same patient at different times (Lowell, 1944a) or either can be present independently. The antibodies associated with insulin-resistance have been described as insulin-"neutralizing" or insulin-"binding" antibodies and are thought to be of the "blocking" type (Loveless & Cann, 1955). The account which follows is mainly concerned with these antibodies, their detection, characteristics and importance.

1. Detection of Insulin Antibodies

The serum of insulin-treated animals and human subjects will, in certain cases, inhibit the biological effects of the hormone or combine with it directly.

a. Inhibition of Hormonal Activity

Amongst other effects, insulin induces hypoglycaemia in experimental animals in vivo and stimulates metabolic processes in isolated tissues in vitro. When mixed with the serum of certain animals and human patients who have received insulin therapy, the hormone fails to produce these effects.

Inhibition in vivo. At first rather crude attempts were made to show that serum from insulin-resistant diabetic patients would inhibit the hypoglycaemic effects of insulin. Thus, when rabbits were used, either no inhibition was observed (Wiener, 1938; Martin, Martin, Lyster & Strouse, 1941; Berne & Wallerstein, 1950) or else resistance to parenteral insulin developed some time after the serum injection (de Wesselow & Griffiths, 1936; Glen & Eaton, 1938; Marble, Fernald & Smith, 1940). In these cases, therefore, factors other than the presence of insulin-neutralizing antibodies were thought to be responsible for insulin-resistance. When mice were first employed (Banting, Franks & Gairns, 1938), serum from an insulin-resistant non-diabetic schizophrenic patient protected them when mixed before injection with an otherwise convulsive dose of the hormone. Later experiments showed that, in mice, serum from insulin-resistant diabetic patients would prevent convulsions (Colwell & Weiger, 1956; Yankelowitch, Massry & Gitter, 1956), inhibit hypoglycaemic symptoms (Lowell, 1944b) or reduce the hypoglycaemic effect (Lowell, 1947; De Filippis & Iannaccone, 1952; Eskind, Franklin & Lowell, 1953; Kaye, McGarry & Rosenfeld, 1955; Loveless & Cann, 1955) of simultaneously injected insulin. None of these protective or insulin-neutralizing effects was produced by serum from insulin-sensitive patients, whether these were diabetic or not. Fractional precipitation of the proteins of such active sera showed that the insulin-neutralizing component was associated with the globulins (Banting *et al.* 1938; Colwell & Weiger, 1956), and by starch-block electrophoresis it was more specifically located in the γ-globulins (De Filippis & Iannaccone, 1952; Sehon, Kaye, McGarry & Rose, 1955). It was also shown to be non-dialysable (Banting *et al.* 1938; Colwell & Weiger, 1956) and thermostable (Lowell, 1942). In one case (Lowell, 1947) the insulin-neutralizing potency of the serum was 0.6 unit/ml.

Insulin-resistance has also been induced in rabbits, guinea-pigs and other animals by means of repeated subcutaneous injections of insulin in Freund's adjuvant (Franklin & Lowell, 1949; Lowell & Franklin, 1949; Moloney & Coval, 1955). Serum from animals treated with insulin by this and other similar methods will also neutralize the hypoglycaemic effect of simultaneously injected insulin in mice (Moloney & Coval, 1955; Arquilla & Stavitsky, 1956b; Lacy & Davies, 1957; Moloney & Goldsmith, 1957; Wright, 1959a), and again the active component is confined to the globulin fraction of the serum proteins and is non-dialysable (Moloney & Coval, 1955). The insulin-neutralizing potency (up to 3.2 units/ml.) of serum from guinea-pigs treated with ox insulin or pig

insulin is greater than that (less than 1 unit/ml.) of serum from other animals such as the rabbit, sheep, horse or mouse (Moloney & Goldsmith, 1957).

Inhibition in vitro. The rate of glucose consumption by, or of glycogen deposition in, the isolated rat diaphragm is stimulated by the addition of insulin to the incubation medium (Gemmill, 1939, 1941). Serum from guinea-pigs treated with bovine insulin in Freund's adjuvant will inhibit the effect of insulin upon glucose consumption by this tissue (Wright, 1959a), whilst serum from some insulin-resistant diabetic patients abolishes the effect of the hormone upon glycogen deposition (Marsh & Haugaard, 1952; Yankelowitch *et al.* 1956).

b. Insulin Binding

All the conventional methods have been used in attempts to demonstrate antibodies in the serum of insulin-treated animals and diabetic patients who are allergic or resistant to insulin (Wasserman *et al.* 1940). The most significant results have been obtained in attempts to show that such serum will precipitate the hormone or combine with it directly in solution or when conjugated with erythrocytes.

Precipitation. No consistent success has been achieved in attempts to precipitate the hormone from solution with serum from insulin-resistant diabetic patients (Goldner & Ricketts, 1942; Berne & Wallerstein, 1950; Davidson & Eddleman, 1950). Usually the serum of insulin-treated animals either fails to precipitate the hormone (Arquilla & Stavitsky, 1956a; Moloney & Aprile, 1959) or produces inconclusive results (Wasserman *et al.* 1940). One notable exception, however, was the serum of one horse which had been treated with bovine insulin; this precipitated various insulins from solution to produce floccules from which the antigen and antibody could be recovered by conventional methods (Moloney & Aprile, 1959).

Binding of labelled insulin. When ^{131}I-labelled insulin is injected intravenously into human subjects, whether diabetic or not, who have received insulin therapy for more than about six months, it disappears from the blood stream more slowly than in the case of untreated subjects (Berson, Yalow, Bauman, Rothschild & Newerly, 1956; Welsh, Henley, Williams & Cox, 1956; Kalant, Gomberg & Schucher, 1958). In the case of insulin-treated subjects, paper electrophoresis of serum obtained shortly after such an injection shows that the labelled insulin is associated with the γ-globulin fraction of the serum proteins (Berson *et al.* 1956; Kalant *et al.* 1958). When labelled insulin is added to whole serum before fractionation by precipitation or electrophoresis, or to the individual protein components after such fractionation, it is bound only by the γ-globulins (Weiger & Colwell, 1956; Burrows, Peters & Lowell, 1957; Kalant *et al.* 1958) or the proteins in the "inter γ–β region" of globulins (Berson & Yalow, 1957b). By contrast, in the case of untreated subjects, labelled insulin, whether injected in vivo or added in vitro, either does not move on paper electrophoresis and becomes bound to the paper at the point of application of the serum, or else under suitable experimental conditions it shows a mobility comparable with the slower-moving albumin or fast-moving α_1-globulin fractions. Ultracentrifugation of serum to which labelled insulin has been added also shows that the hormone moves with the γ-globulins in the case of insulin-treated patients, but with the albumin fraction of serum from untreated patients (Berson *et al.* 1956). By comparing the rates at which labelled insulin is removed from the blood stream after injection (Welsh *et al.* 1956; Kalant *et al.* 1958), or, with more precision, by determining the amount of labelled insulin which, when added in vitro, is bound by the γ-globulins (Weiger & Colwell, 1956; Burrows *et al.* 1957; Berson & Yalow, 1959a), the insulin-binding capacities of such sera have been estimated. In the latter case the amount of labelled insulin added in vitro that moves on electrophoresis with the γ-globulins is measured, but alternatively the γ-globulins and labelled insulin associated with them can be precipitated with anti-human γ-globulin prepared in rabbits (Skom & Talmage, 1958a, 1958b). No correlation has been established between the insulin requirements of insulin-sensitive diabetic patients and the insulin-binding capacities of their sera; usually not more than 20 units of insulin are bound by the γ-globulins in a litre of such sera. However, amongst insulin-resistant diabetics requiring more than 200 units/day over long periods, the serum insulin-binding capacities are usually high (Weiger & Colwell, 1956) and capacities of the order of 500 units/l. or more have been reported (Burrows *et al.* 1957; Berson & Yalow, 1959a). As in the case of the insulin-neutralizing component of the serum proteins, the insulin-binding substance is non-dialysable.

Agglutination of insulin-conjugated erythrocytes. After treatment with tannic acid or bisdiazobenzidine, erythrocytes can be conjugated with insulin and are then agglutinated by serum from rabbits (Arquilla & Stavitsky, 1956a; Moinat, 1958) or from diabetic patients (Arquilla & Stavitsky, 1956a; Yankelowitch *et al.* 1956) receiving repeated injections of insulin. In the case of rabbit serum, the active component is associated with the globulins.

2. Characteristics of Antibodies

The active components of serum responsible for insulin neutralization and binding fulfil the first condition of the definition of an antibody as quoted on p. 219, since they appear only when insulin treatment has been administered. There is no evidence at present to suggest that they do not react specifically with the antigen. Agglutination of insulin-conjugated red cells is not inhibited by non-specific proteins (Arquilla & Stavitsky, 1956b; Moinat, 1958), and serum from insulin-treated guinea-pigs does not inhibit the stimulant effects of synthalin and phenethyldiguanide (synthetic hypoglycaemic agents) upon glucose consumption by the isolated rat diaphragm (Wright, 1959a). Formal proof of their specificity for the antigen insulin is still needed, but all the available evidence suggests that the insulin-neutralizing and insulin-binding substances are antibodies. Whether they are identical with one another is also not certain, but in several instances serum which has proved active as a hormone inhibitor has also shown insulin-binding properties (Arquilla & Stavitsky, 1956b; Weiger & Colwell, 1956; Welsh *et al.* 1956; Yankelowitch *et al.* 1956; Burrows *et al.* 1957; Kalant *et al.* 1958; Moloney & Aprile, 1959).

Though probably specific in their reactions with the hormone, insulin-binding and insulin-neutralizing antibodies do not show any marked degree of species specificity. Thus serum from animals or human subjects treated with insulin from one animal source will react with insulin extracted from the pancreas of other animal species. In the case of serum of animals treated with bovine or pig insulins, this has been demonstrated in mice (Moloney & Coval, 1955; Moloney &

Goldsmith, 1957; Moloney & Aprile, 1959), the isolated rat diaphragm (Wright, 1959a) and insulin-conjugated red cells (Arquilla & Stavitsky, 1956b). Berson & Yalow (1959b) have demonstrated slight species differences in insulin-binding capacity, using serum from insulin-treated diabetic patients; bovine and sheep insulins are bound slightly more firmly than pig and horse insulins. They suggest that this may be because of the differences which are known to exist in their chemical compositions (Harris, Sanger & Naughton, 1956). They could find no evidence to support the findings of Lowell and his co-workers (Burrows *et al.* 1957), that insulin-resistant diabetics produce insulin-binding and insulin-neutralizing antibodies that are specific for the hormone used in therapy and do not react with human insulin. It has been shown, however, that mice are not protected from the effects of guinea-pig insulin by the serum of animals treated with bovine or pig insulins (Moloney & Coval, 1955). The guinea-pig does produce potent anti-insulin serum, and insulin extracted from its pancreas behaves differently from bovine and sheep insulins on chromatography (Goldsmith & Moloney, 1957). It is possible, therefore, that if only slight changes in chemical composition accompany minor immunological differences, the unique immunological behaviour of guinea-pig insulin may be due to a marked difference in its chemical composition.

Berson & Yalow (1957a, 1957b, 1957c, 1959a) have studied the kinetics of the reaction between insulin and the antibody present in serum from insulin-treated patients. The results suggest that one molecule of the antibody binds two molecules of the antigen, one of which is released at a much more rapid rate than the other. The reversibility of this reaction, which has been noted by others (Kalant *et al.* 1958; Skom & Talmage, 1958a), implies that the antibody does not inhibit the action of the hormone but rather prolongs its period of action. It was thought at one time that the antibody inhibited insulinase activity in the liver and might so reduce the rate of insulin destruction in the body (Williams, 1956), but Yalow & Berson (1957) showed that this inhibitory effect observed in vitro was due to the slow release of insulin bound by the serum antibody and not to inhibition of any enzyme. For these reasons Berson *et al.* (1956) originally suggested that the insulin-binding antibody should be described as an "insulin-transporting antibody"; it delays the passage of exogenous insulin to the tissues but does not interfere with its biological action.

These antibodies associated with the serum γ-globulins and probably of the "blocking" type (Loveless & Cann, 1955) must be distinguished from insulin antibodies of the "reagin" type responsible for insulin allergy, and from other insulin antagonists which have been found in serum. The antibodies responsible for skin reactions in diabetic patients are associated with the β-globulins (Loveless & Cann, 1955), are thermolabile (Lowell, 1942) and do not necessarily appear in the serum of insulin-resistant patients. The insulin antagonist described by Vallance-Owen, Dennes & Campbell (1958) occurs in the plasma of normal subjects as well as in that of diabetics, and its appearance is not a result of insulin therapy; it is found in the albumin fraction of the plasma proteins. Field, Tietze & Stetten (1957) have also described an insulin antagonist which appears during diabetic acidosis; it is associated with the α_1-globulins and its presence is not evoked by insulin therapy. Other insulin antagonists have been reported in diabetic animals and patients, but these also are distinct from the antibodies occurring in insulin-treated patients and animals (see the paper on insulin antagonists by Vallance-Owen on p. 214 of this number of the Bulletin).

3. Practical Significance of Antibodies

As already emphasized, insulin-binding and insulin-neutralizing antibodies appear in large amounts in the serum of some insulin-resistant diabetics and give rise to a rare but important clinical problem. In addition, however, these antibodies induced in the guinea-pig have been used to locate or identify insulin in biological material and to produce an insulin-deficient state in experimental animals.

a. Insulin-Resistant Diabetics

In the small proportion of insulin-resistant diabetics in whom insulin-binding or insulin-neutralizing antibodies have been demonstrated in the serum, very large doses of insulin are needed to prevent ketonuria or reduce glycosuria. If, as Berson & Yalow (1959b) suggest, the antibody combines reversibly with the hormone and does not otherwise interfere with its biological activity, then, so long as resistance is present, the patient should respond normally if sufficient insulin is given to neutralize most of the circulating antibody; but in addition, should resistance cease rapidly, then large amounts of circulating insulin bound to the antibody would be released to produce severe and persistent hypoglycaemia. In fact Smelo (1948) has already pointed out that diabetes in such patients can be controlled satisfactorily during these resistant phases, provided that sufficient insulin is given for a sufficient period of time. In addition, the danger of the sudden onset of severe hypoglycaemia in an insulin-resistant patient is not unknown; Eskind *et al.* (1953) describe a fatal case. Although high insulin-binding capacities are associated with such insulin-resistance, Berson & Yalow (1959b) do not consider that the presence of these antibodies per se is entirely responsible for the condition; the rate of antibody production and destruction, the amount of free insulin needed by the tissues to maintain normal metabolism, and the rates of formation and dissociation of the antigen–antibody complexes may also determine the dose of insulin needed to control the patient's condition. Thus it can be said that a relationship has been established between insulin-resistance and insulin-binding antibodies in some cases but that much has still to be learned concerning the part played by the antibodies.

b. Identification of Insulin in Biological Material

Serum from guinea-pigs treated with insulin has been used to identify the hormone in blood plasma and its active protein fractions (see the paper on insulin in blood by Randle & Taylor on p. 209 of this number of the Bulletin), in the tissues of a murdered woman, and in the islet tissue of the pancreas.

In 1957, a woman was found dead in her bath and apparently drowned. Her husband was accused of having injected her with insulin, and injection marks were found on the buttocks. Extracts prepared from the tissues underlying these injection marks produced biological effects in vivo and in vitro which were similar to those of the hormone. The stimulant effects of these extracts, like those of insulin (Wright, 1959a), upon the isolated rat diaphragm were abolished by serum from insulin-treated guinea-pigs but were unaffected by serum from normal guinea-pigs. This evidence, together with the fact that

one or other of the biological effects was abolished or reduced after treatment of the extracts with cysteine, rat-liver extracts and pepsin, provided information sufficient to show that the tissues removed from the dead woman contained insulin (Birkinshaw, Gurd, Randall, Curry, Price & Wright, 1958).

There is now sufficient evidence to establish that insulin is produced in the pancreas by the β cells of the islets of Langerhans (see Lazarow, 1957). The hormone has been demonstrated in these cells by means of an aldehyde fuchsin stain (Gomori, 1950) and a reagent which is known to react with thiol and disulphide groups (Barrnett, Marshall & Seligman, 1955). Insulin contains disulphide groups. Lacy & Davies (1957) have since shown that the globulin fraction of serum from guinea-pigs treated with bovine or pig insulins, when coupled with fluorescein, will stain the islet tissue of freeze-dried sections of pancreas from cattle, the cat, the mouse and the rat but not that of the guinea-pig, rabbit or man. Their failure to demonstrate reactions in the islet tissue of man has not yet been explained and could have been due to technical errors. They have now improved the technique, which has been reported in detail (Lacy & Davies, 1959), but the differentiation of cell types within the islets has not yet been satisfactorily accomplished.

c. Production of Insulin Deficiency in Experimental Animals

Various methods have been adopted for the production of diabetic syndromes in experimental animals (see Danowski, 1957), and all involve the destruction or removal of the insulin-secreting cells of the pancreas. Some are technically difficult or impossible, and the insulin deficiency syndromes are all complicated to a greater or lesser degree by the effects of other lesions. However, Moloney & Coval (1955) showed that serum from guinea-pigs treated with bovine and pig insulins induced hyperglycaemia on intravenous or intraperitoneal injection into mice. Repeated injections produced glycosuria, ketonuria and some loss of body-weight. Subsequently Moloney & Goldsmith (1957) reported that the best effects were obtained when mice were injected intravenously or intraperitoneally with potent anti-insulin sera (3.2 units/ml.). Other experiments have been reported (Wright, 1959b; Armin, Grant & Wright, 1960a, 1960b) in which serum from guinea-pigs treated with bovine insulin has been injected or infused intravascularly into experimental animals. Single injections produce transient hyperglycaemia in the rat, rabbit and cat but have no effect upon the blood-glucose concentration of the guinea-pig; serum of normal guinea-pigs is inactive. The rate at which the blood-sugar concentration rises in the early stages in the rat and rabbit (3.16 mg./100 ml./min.) suggests that glucose is entering the extracellular space at the rate of approximately 0.8 mg./100 g. body-wt./min., which is less than the glucose turnover rate (1.67 mg./100 g. body-wt./min.) reported for normal rats (Feller, Strisower & Chaikoff, 1950). The hyperglycaemic effect is immediate and can be prolonged in the rat either by continuous intravenous infusion (20 hr.) or by administration of larger doses intraperitoneally. If the dose injected or the rate of infusion is sufficient, the blood-sugar concentration rises within an hour to about 300 mg./100 ml. and remains at this level, provided that the animal receives sufficient fluid. Heavy glycosuria associated with polyuria occurs in 1–2 hr. and is followed 4–5 hr. later by ketonuria, both of which persist until normoglycaemia is re-established. Within 10 hr. rats injected intraperitoneally develop marked weakness, oliguria and increased hyperglycaemia, unless fluid is administered subcutaneously. This dehydration and oliguria, which themselves may prove fatal, can be avoided by infusing both serum and saline intravascularly. Under these conditions rats may remain diabetic for 2–4 days, after which most die in a wasted acidotic anuric state, whilst some may recover spontaneously. This syndrome is very similar to that occurring in the juvenile type of human diabetics (Lawrence, 1951) and in the "totally" depancreatized rat (Scow, 1957) but differs in some respects from alloxan diabetes in the rat (see Lukens, 1948). Technically it is a syndrome which is simple to produce and, as far as is known, is not complicated by any undesirable lesions. Animals prepared by this method are perfectly normal before the administration of the anti-insulin serum, and their carbohydrate metabolism is restored to normal if the dose is not too great. Hence changes in tissue metabolism which accompany the rapid onset and subsequent remission of this syndrome can be studied under controlled conditions; this has not been possible with experimental diabetes induced by any other method.

4. Comment

As Berson & Yalow (1958, 1959b) point out, the insulin-neutralizing and insulin-binding antibodies may or may not be identical but there is no doubt that they antagonize the hormone. They undoubtedly play a part in the production of some cases of insulin-resistance, though much still remains to be learned of their contribution to the syndrome. Antibody production in animals provides a more satisfactory method for studying the antigenic properties of the hormone, since purified preparations from various sources are now available or could be produced. Already it is apparent that the guinea-pig is unique in this respect, but little is known of the structure of the insulin molecule which it produces. There may be other related species (e.g., the capybara) which also produce insulins differing markedly in immunological and possibly chemical properties from those of bovine, pig and sheep insulins. It may be that serum from guinea-pigs treated with bovine insulin could be injected into other animals which might not then become hyperglycaemic, and might therefore be producing insulins which also differ markedly from those mentioned above. The problem may not be as simple as this, for Moloney & Coval (1955) have already suggested that insulin extracted from the pancreas of an animal (altered insulin) may be affected by the chemical process involved and thus differ from the hormone secreted into the blood stream (endogenous insulin). Also serum from one animal may show different immunological properties from that obtained from another treated with insulin from the same third animal source; serum from guinea-pigs treated with bovine insulin produces hyperglycaemia in mice, whereas serum from similarly treated horses does not (Moloney & Goldsmith, 1957). Problems such as these abound, and accurate methods for the assay of insulin-binding antibodies are now available. It therefore seems that, if the methods which have been used so effectively in the study of insulin antibodies in human subjects could now be applied to antibodies produced in experimental animals, significant advances could be made. In addition, the sera which have been used to produce hyperglycaemia in experimental animals should be more rigorously characterized from the immunological point of view. To date it has been assumed, on the basis of arguments outlined above, that these sera

contain insulin-specific antibodies and that therefore the syndromes which they induce are due to insulin deficiency. This requires formal confirmation, but meanwhile the insulin-deficient syndrome should be further investigated and compared with those encountered in other forms of experimental and human diabetes.

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THE ACTION OF INSULIN ON CARBOHYDRATE METABOLISM

R. B. FISHER M.A. B.Sc. D.Phil.

Department of Biochemistry
University of Edinburgh

1 Interaction of hormones in carbohydrate metabolism
2 Effects of insulin on the carbohydrate metabolism of the liver
3 Action of insulin on peripheral tissues
4 Alternatives to the hexokinase hypothesis
References

The early observations on the effects of pancreatectomy, pancreatic duct ligation and obstruction, and pancreatic transplantation led to the belief that the islet tissue secreted a hormone, first called insulin by de Meyer in 1909, which regulated the concentration of glucose in the blood. The belief that absence of this hormone was responsible for the disabilities of diabetes mellitus was already generally held before the hormone became available.

As soon as a sufficiently pure concentrate of insulin was prepared by Banting & Best (1922) it became evident that it could indeed maintain at a normal level the blood sugar of a depancreatized animal and could reduce the blood sugar of a normal animal.

Since that time much work has been directed toward the explanation of these effects. The major possible sites of action on carbohydrate metabolism have been taken to be the liver, the major glycogen-forming and glycogen-storing organ, and the skeletal muscles, the major carbohydrate-consuming tissue.

1. Interaction of Hormones in Carbohydrate Metabolism

Most early work directed to the solution of these problems was carried out on intact or almost intact animals, and is now difficult to interpret, in view of the more lately acquired knowledge of the interaction of hormones in carbohydrate metabolism. The same difficulties of interpretation may still obscure the significance of modern work on the effects of insulin on the liver.

Himsworth (1934) and Himsworth & Scott (1938) showed that the intravenous glucose tolerance of rabbits was increased by a high-carbohydrate diet and lowered by a high-fat diet, and confirmed the finding of earlier workers that repeated injections of small amounts of glucose at short intervals produced an increasing glucose tolerance. They also showed that a high-carbohydrate diet or repeated small glucose injections given over some hours produced an increase in insulin-sensitivity. These effects were not observed in hypophysectomized animals. The reasonable conclusion was that some pituitary antagonist to insulin was secreted at a rate which was decreased by an increase in the rate of carbohydrate metabolism.

It is well known also that lowering of the blood-sugar concentration can cause adrenaline secretion, and further work on this topic has recently been reported by Armin & Grant (1959). Since it is believed that adrenaline can cause secretion of adrenocorticotropic hormone and that adrenal corticosteroids can stimulate hepatic gluconeogenesis (Long, 1952), the number and variety of the effects on carbohydrate metabolism consequent on rise in concentration of circulating adrenaline are very large.

A vivid example of the impact of secondary factors on insulin effects in the intact animal is afforded by the work of Hastings and his colleagues. Renold, Hastings, Nesbett & Ashmore (1955) showed that, if liver removed from rats with diabetes induced by alloxan and receiving continuous insulin treatment at different times after commencement of the treatment was studied biochemically, the various impairments in its metabolic activities that had been established in the diabetic animals did not begin to disappear until more than 24 hours had elapsed, although the blood sugar was brought within normal limits in a matter of minutes. This work strongly suggests that the defects in liver metabolism were the outcome of secondary consequences of insulin deficiency not directly corrigible by the provision of insulin itself.

2. Effects of Insulin on the Carbohydrate Metabolism of the Liver

The basis of the belief that insulin has an effect on the liver is to be found in the work of Cori (1925), Cori & Cori (1928), Corkill (1930) and Goldblatt (1930). It is undoubtedly the case that in some species in some conditions the injection of insulin can give rise to changes in liver-glycogen content. But in some instances a rise in liver-glycogen content has been observed, in others no change, and in others a fall in glycogen, and there can be no doubt that secondary consequences of insulin injection play a large part, and perhaps an exclusive part, in the responses observed.

Lundsgaard, Nielsen & Orskov (1936) failed to find any effect of insulin on perfused cat liver. The topic was revived more recently by the work of Bearn, Billing & Sherlock (1951) and Bouckaert & de Duve (1947). Sherlock's work depended on the measurement of differences in glucose concentration between arterial and hepatic venous blood in human subjects, but depended for its interpretation on the arbitrary assumption that insulin had no effect on the glucose uptake of the tissues nourished by the primary capillaries of the portal circulation. On this basis, the observation that insulin increased the difference between the glucose content of the arterial blood supply and that of the venous outflow was interpreted to mean that insulin suppressed hepatic glucose output.

In the experiments of de Duve it was shown that the rate of glucose infusion needed to maintain constant blood-glucose concentration in a dog receiving a maximal dose of insulin was very much reduced when the liver was rapidly removed from the circulation. It was concluded that two-thirds of the increase in glucose consumption brought about by insulin was caused by increased hepatic consumption of glucose. However, Lang, Goldstein & Levine (1954) showed that hepatectomy depressed the effect of insulin on the glucose uptake of the hind limbs of dogs to an extent sufficient to account for the findings of Bouckaert & de Duve (1947). It does not seem necessary to assume that insulin has a direct effect on the liver to account for these findings.

More recently several groups of workers have attempted to determine whether insulin regulates hepatic glucose output, by methods utilizing labelled glucose. Several groups have described independently the effects of insulin on the specific activity of blood glucose. There is, in general, agreement that it diminishes exponentially. Searle & Chaikoff (1952) found that, when insulin is injected, there is a period in which the specific activity ceases to fall. This has been interpreted on the assumption that the fall before administration of insulin is due to dilution of injected labelled glucose by the hepatic glucose output, the cessation of fall then denoting cessation of hepatic glucose output. Shoemaker, Mahler & Ashmore (1959) have measured the differences in concentration of glucose between the blood of the portal vein and that of the hepatic vein, and between the blood of the hepatic artery and that of the hepatic vein, together with the blood flow in the portal vein, and have shown that the hepatic glucose output is not decreased by insulin. On the other hand, the total splanchnic glucose output is decreased, strongly suggesting that the assumption of Bearn *et al.* (1951), that the splanchnic organs could be neglected, is in error. Though Shoemaker *et al.* (1959) observed that insulin induced an interruption in the fall in specific activity of blood glucose under the conditions of their experiments, it seems most likely that this fall is not due to a cessation in the output of glucose by the liver. Steele (1959) has suggested that observations made by Steele, Wall, de Bodo & Altszuler (1956) may provide an alternative explanation of the phenomenon. These workers showed that injected labelled glucose mixed relatively slowly with a significant part of the pool of body glucose. The effect of such a lag would be that after a time the "slow" part of the pool would have a higher specific activity than the circulating blood glucose, and any factor tending to increase exchange between it and the blood glucose would diminish the rate of fall of specific activity. Coxon & Robinson (1959) showed that this kind of effect occurred when labelled bicarbonate was injected into dogs. Shortly after the injection, the difference in specific activity of the bicarbonate in the blood of the femoral artery and that in the femoral vein was less than unity, but after 10–40 minutes it became markedly greater than unity.

Steele (1959) obviated effects due to such lags in equilibration by injecting trace amounts of labelled glucose sufficient to maintain constant blood-glucose specific activity, so that the only factor likely to be able to alter the specific activity was the rate of hepatic output of unlabelled or relatively unlabelled glucose. When insulin was given in these conditions, it did not alter blood-glucose specific activity.

Martin, Leonards & Miller (1959) have shown that the rate of fall of blood sugar is regularly greater when a particular dose of insulin is given into a peripheral vein than when it is given into the portal vein. This makes it unlikely that the liver is a major site of any blood-sugar-lowering effect of insulin.

Thus, in general, the evidence points away from the liver as a major site of insulin action, but in view of the difficulty of setting up an isolated liver preparation which can be regarded as effectively normal except for the absence of insulin, and in view of the known secondary effects which follow deprivation of or provision of insulin in vivo, the question of the existence of an insulin effect on liver cannot be regarded as settled. There are such observations as that of Brady & Gurin (1950), who showed that the incorporation of labelled acetate into the fatty acids of liver slices was accelerated by insulin in the presence of glucose, but not by glucose or insulin alone, an observation exactly similar in nature to that of Balmain, Folley & Glascock (1952) on the incorporation of acetate into the fatty acids of mammary gland slices. The concentration of insulin necessary to produce this effect in liver was exceedingly high, but since liver is known to destroy insulin very rapidly, this cannot be taken as a basic objection to the experiments.

It must be supposed, therefore, that insulin may have an action on liver carbohydrate metabolism. This action is unlikely to be one of grossly increasing the glucose uptake of the liver, as de Duve believed, and it is unlikely to be one of directly inhibiting glucose output. It may even be that the explanation of the findings of Brady & Gurin (1950) is to be found in a direct action of insulin on fat metabolism, the process affected being one which is dependent on simultaneous glucose metabolism.

3. Action of Insulin on Peripheral Tissues

The term "peripheral tissues" is an awkward one. Like many physiological terms, it has outlived its usefulness but is difficult to replace. So long as one could consider the liver as the major carbohydrate supplier, and the skeletal muscles as the major glucose consumer, it was useful to describe skeletal muscle and other limb and trunk tissue as peripheral tissues. But cardiac tissue and extra-hepatic splanchnic organs do not fit happily into this category of peripheral tissue. Nevertheless it will be most satisfactory to continue to use the term, connoting by it all glucose-consuming organs.

The first study of the action of insulin on an isolated organ was that of Hepburn & Latchford (1925), who showed that it markedly increased the glucose uptake of the perfused rabbit heart. Best, Dale, Hoet & Marks (1926) showed that insulin increased the rate of glucose oxidation and the rate of glycogen deposition in the muscles of the decapitated eviscerated cat, and later Gemmill (1933) showed that it increased the glycogen deposition in the isolated rat diaphragm.

By far the greater part of work on the action of insulin on the carbohydrate metabolism of isolated organs has been performed on the rat diaphragm. The apparent utility of this preparation for insulin bio-assay has resulted in very large numbers of observations, but there seems to have been little work on the relations between rate of glucose uptake and rate of glycogen formation. Perlmutter & Greep (1948) and Li, Kalman & Evans (1949) measured glucose uptake and glycogen formation simultaneously. The findings of both groups indicate that glucose uptake was increased more than was accountable for by increase in glycogen synthesis. On the other hand, insulin has been shown not to increase the oxygen uptake of the diaphragm, so that the fate of the glucose not converted to glycogen is not certain.

However, Winegrad & Renold (1958) have shown with the aid of labelled glucose that insulin increases the rate of glucose catabolism in the epididymal fat pad of the rat, as well as accelerating anabolic processes, e.g., the incorporation of glucose carbon into the fatty acids of the tissue.

Thus the view that insulin influences some very early process in the chain of intracellular transformations of glucose seems to have a sound basis.

Cori (1946) concentrated attention on the one enzymically catalysed reaction common to the paths leading to glycogen on the one hand and to the catabolism of glucose on the other,

namely the hexokinase reaction, and was unable to show that insulin activated this process. Price, Cori & Colowick (1945), Price, Slein, Colowick & Cori (1946) and Colowick, Cori & Slein (1947) believed, however, that they had shown that insulin could remove an inhibitory effect on this reaction brought about by an anterior pituitary factor. Stadie & Haugaard (1949) were unable to repeat these results, and they do not seem to have been reproduced by any other worker. Perlmutter & Greep (1948) were able to show that insulin has as large an effect in vitro on the diaphragm of hypophysectomized rats as on normal rats; this is inexplicable if the action of insulin is to inhibit a pituitary depressant of carbohydrate metabolism.

Accordingly, it appears that there is no ground for supposing that insulin activates hexokinase or removes a physiological inhibition of this enzyme. If, therefore, an explanation of insulin action on carbohydrate metabolism is to be found which will account for acceleration of anabolic and catabolic pathways of carbohydrate metabolism, it appears at first sight that it must be looked for at an earlier stage than the hexokinase reaction.

4. Alternatives to the Hexokinase Hypothesis

It is possible that the catabolism of glucose could furnish the energy for the anabolic transformation of further glucose, so that the sole action of insulin on carbohydrate metabolism could be conceived as being an activation of glucose catabolism. The work of Balmain *et al.* (1952) on the mammary gland, of Brady & Gurin (1950) on the liver, and of Winegrad & Renold (1958) on the epididymal fat pad shows that insulin accelerates fat synthesis from acetate only when glucose is supplied, strongly suggesting that the insulin effect on fat anabolism is dependent on its action on carbohydrate catabolism.

The finding of Krebs & Eggleston (1938) that insulin inhibited the falling-off in oxygen consumption of pigeon breast muscle therefore still has an interest, despite the fact that conditions for the production of this effect in any other tissue preparation have not so far been found.

Chain and his colleagues, in work summarized by Chain, Beloff-Chain & Pocchiari (1956), applied quantitative radiochromatographic methods to the study of the effect of insulin on the carbohydrate metabolism of the isolated diaphragm. Their main conclusion was that as insulin altered the pattern of metabolism qualitatively, it was not likely that its action was chiefly on the access of glucose to the cells: it must modify specifically some intracellular metabolic process. However, these authors have treated the diaphragm as though it were homogeneous, taking no account of the existence in it of a considerable extracellular space. Nor have they taken account of the findings of other workers, which show that appreciable quantities of some enzymes leak out of the cells of the diaphragm. That such enzymes can catalyse some partial reactions of carbohydrate metabolism which occur at a significant rate has been shown by Candela, Sols, Alvarado, Santiago, Villar-Palasi & Candela (1957).

Beloff-Chain, Chain, Bovet, Pocchiari, Catanzaro & Longinotti (1953) had shown that isolated rat diaphragm is capable of metabolizing glucose 1-phosphate and glucose 6-phosphate. Candela *et al.* (1957) were able to show that the rate of disappearance of glucose 1-phosphate from the medium in which diaphragm had previously been incubated was as high as the rate in the presence of the diaphragm, and were able to demonstrate in the medium the enzymes in concentrations adequate to account for the transformation of the ester. Zierler (1956) has independently shown that aldolase is continually escaping from diaphragm and believes that this escape occurs across the boundaries of intact cells.

Since, then, appreciable quantities of the metabolites found by Chain *et al.* (1956) in the diaphragm must be taken to be extracellular, and since it appears likely that some kind of extracellular metabolism is going on in such preparations in addition to intracellular metabolism, there is still doubt whether the change in pattern of metabolism found by these workers implies any more than a change in the proportions in which intracellular and extracellular transformations are taking place. Such a change in proportion would be expected to occur if the major action of insulin was to govern the rate of access of glucose to the interior of cells.

One other series of observations gives an indication that insulin might stimulate preferentially particular pathways in the intracellular metabolism of glucose, namely those of Winegrad & Renold (1958). These workers studied metabolism of glucose U-^{14}C, glucose 1-^{14}C and glucose 6-^{14}C in the epididymal fat pad of the rat, and the changes brought about by insulin. The partition of ^{14}C between CO_2 production, incorporation into lipid, lactate formation and undetermined pathways is not affected in the U-^{14}C experiments, but insulin brings about a greater production of $^{14}CO_2$ from glucose 1-^{14}C than from glucose 6-^{14}C. This might be taken to indicate preferential stimulation of metabolism by the phosphogluconate pathway, but it seems more likely that it merely signifies lesser dilution of C-1 when metabolized by this pathway than when metabolized by the Embden-Meyerhof pathway. Winegrad & Renold (1958) themselves come to the conclusion that in this tissue the stimulatory effect of insulin is exercised equally on both pathways.

This work is of considerable importance. Although the analysis of the fate of glucose is not so detailed as that of Chain *et al.* (1956), it does suggest quite strongly that insulin does not here alter the pattern of carbohydrate metabolism, despite a very large effect on its rate. This in turn suggests that the finding of Chain concerning alteration of metabolic pattern of glucose by insulin is not necessarily a general phenomenon, and therefore probably not a secure basis on which to build a hypothesis of insulin action.

Thus, in general, the action of insulin on carbohydrate metabolism remains difficult to define. The evidence for any of the actions proposed for it in the liver is insecure. It seems certain that it can increase the glucose uptake of peripheral tissues. In one or other tissue it has been shown to increase glycogen formation, conversion of glucose to fat, lactate formation and glucose oxidation to CO_2, and the indication in Winegrad and Renold's work is that it stimulates catabolism by the phosphogluconate pathway to the same extent as catabolism by the Embden-Meyerhof pathway. So the balance of evidence is in favour of an action at the earliest possible point in cellular metabolism. Since the hypothesis that this point of action is at the hexokinase stage has had to be abandoned, the most likely site of action is on the processes governing the entry of glucose into the cell.

The great mass of work stemming from the original demonstration by Levine and his colleagues (see Levine, Goldstein, Huddlestun & Klein, 1950) that insulin affects the permeability of cells to sugars is dealt with by Randle & Young in

this symposium (p. 237) and there is no need here to do more than to make the comment that if it be true that the major action of insulin in relation to carbohydrate metabolism is to regulate the access of glucose to peripheral cells, then in the biochemical sense it may well have no action at all on carbohydrate metabolism.

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INSULIN AND METABOLISM OF FATTY ACIDS

S. J. FOLLEY Ph.D. D.Sc. F.R.S.

Physiology Department
National Institute for Research in Dairying
Shinfield, Reading

A. L. GREENBAUM Ph.D. D.Sc.

Department of Biochemistry
University College, London

1 Liver tissue
2 Mammary gland
3 Adipose tissue
4 Insulin and the dependence of lipogenesis on carbohydrate metabolism
5 Synoptic discussion
References

This paper is restricted to a discussion of the influence of insulin on fatty acid metabolism; lack of space precludes any consideration of the hormonal control of triglyceride, phospholipid or cholesterol metabolism.

It has been known for a very long time that diabetes is accompanied by an excretion of ketone bodies, and there is little doubt that this is the result of over-production of acetoacetate by the liver. Thus, Stadie, Zapp & Lukens (1940) found that liver slices from depancreatized cats produced some five times more ketones than did slices from normal livers, while Chaikoff & Soskin (1928) have shown that the rate of removal of acetoacetate from the blood by the peripheral tissues is essentially normal in diabetic dogs. Much of the evidence that the over-production of ketone bodies in diabetes originates from a disordered fatty acid metabolism in the liver has been reviewed by Stadie (1942).

The discovery that the diabetic animal exhibits not only impaired oxidation of fatty acids, but also a defect in the synthesis of fatty acids (lipogenesis) from carbohydrate, stems from the work of Drury (1940) who found that although a diabetic animal could, without insulin, satisfy its energy requirements from a carbohydrate-rich diet, it could not convert carbohydrate to fat under these conditions. The metabolic defect could be made good by the administration of insulin. The importance of this finding was subsequently underlined by the work of Stetten & Boxer (1944) who showed that, while a normal rat could convert about 35% of its dietary carbohydrate to fat, lipogenesis was reduced to vanishing point in the diabetic animal. In the intact animal the action of insulin appears to be antagonized by anterior pituitary factors, principally growth hormone. Thus, the lost ability of liver slices from a depancreatized cat to synthesize fatty acids from acetate could be largely restored if the animal was hypophysectomized (Brady, Lukens & Gurin, 1951). This contributes to the relative mildness of the diabetes characteristic of the hypophysectomized-depancreatized animal.

The role of insulin in lipogenesis has been mainly investigated, by in-vitro studies, on three tissues—liver, mammary gland and adipose tissue—which will be considered in turn. Information on the role of carbohydrate in providing carbon for lipogenesis has mainly come from the latter two tissues. Until the introduction of the epididymal fat pad as an experimental tissue, developed by the work of Renold and his co-workers at Harvard (Winegrad & Renold, 1958a, 1958b), the mammary gland was regarded as one of the most active sites of fatty acid synthesis and has been intensively studied in the laboratory of one of us (S.J.F.) and by Chaikoff and his school at Berkeley, California.

1. Liver Tissue

Twelve years ago, Bloch & Kramer (1948) showed that insulin in vitro stimulated the incorporation of acetate (in the presence of pyruvate) into the fatty acids of liver slices from intact rats. Brady & Gurin (1950b) demonstrated essentially the reverse of this when they found that liver slices from rats with alloxan-induced diabetes showed a markedly reduced ability to incorporate acetate into long-chain fatty acids. Surprisingly, as Chernick & Chaikoff (1950) found, the impaired lipogenesis in liver slices from diabetic rats could not be made good by the addition of insulin to the medium; for this it was necessary to pre-treat the animals with insulin for several days, by which time the capacity of the liver tissue to synthesize fatty acids in vitro had been restored to normal, even when the diabetic state had existed for 150 days. There are thus apparently anomalous features about the in-vitro action of insulin on lipogenesis in liver slices from normal and diabetic rats respectively.

The enzymic site of the failure in lipogenesis in the liver of the diabetic animal has been extensively studied, particularly in the laboratories of Chaikoff and of Gurin at Philadelphia. It has been suggested by Brady & Gurin (1950b) that this failure could not arise from lack of acetyl-coenzyme A because, although lipogenesis from acetate or short-chain fatty acids was completely suppressed in liver slices from diabetic rats, the oxidation of octanoate, which goes through acetyl coenzyme A, proceeded at a normal rate in this tissue. Moreover, cholesterol synthesis, which requires acetyl-coenzyme A, may even be enhanced in liver slices from diabetic animals (Hotta & Chaikoff, 1952). This work thus indicated that the block in fatty acid synthesis in diabetes occurred at some point subsequent to the formation of acetyl-coenzyme A.

At the same time, it had become evident that lipogenesis was closely linked to carbohydrate metabolism. Thus, Bloch & Kramer (1948) found that lipogenesis from acetate in liver slices was accelerated by the presence of carbohydrate in the medium, and similar results for mammary gland were soon afterwards reported (Folley & French, 1950; Balmain, Folley & Glascock, 1952). Investigations on the role of insulin in lipogenesis thus inevitably involved consideration of why an active carbohydrate metabolism was necessary for lipogenesis.

The level at which glucose metabolism is blocked in the diabetic liver and the effect of this block on fat metabolism were investigated by Chaikoff and his collaborators using ^{14}C-labelled glucose, fructose and acetate. It was shown that, while the oxidation of glucose was impaired in liver slices from diabetic animals, that of fructose was normal (Chernick, Chaikoff & Abraham, 1951). The incorporation of fructose

carbon into fatty acids was, however, depressed in diabetic liver. In addition, work done at the same laboratory showed that liver slices from alloxan-diabetic rabbits, which could not oxidize glucose, could oxidize lactate, pyruvate and acetate at rates comparable with those found in liver slices from normal animals, but could not incorporate any of these substrates into long-chain fatty acids. Since the rates of oxidation of fructose, lactate, pyruvate and acetate were essentially normal, it was concluded that the enzyme systems of the Embden-Meyerhof (EM) pathway and of the Krebs cycle from fructose 6-phosphate onwards were intact, and that the initial block in glucose utilization was at the level of the enzymes converting glucose to fructose 6-phosphate, i.e., possibly involving the hexokinase reaction. Moreover, since all the substrates mentioned except glucose could be oxidized by diabetic liver tissue, but not incorporated into fatty acids, a second block in the metabolic pathway from carbohydrate to fatty acids was postulated in the linking of carbohydrate metabolism to fatty acid synthesis (Chernick & Chaikoff, 1951).

The factors which would enable a diabetic animal to circumvent the second block in lipogenesis from carbohydrate were: (i) treatment of the animal with insulin for several days; or (ii) feeding the animal with fructose for several days; in the latter case, although the power to incorporate lactate or acetate into fatty acids was restored, the ability to oxidize glucose or use it for lipogenesis was not. Further evidence of the close connexion between carbohydrate dissimilation and lipogenesis was provided by the finding that lipogenesis is depressed in starvation (Masoro, Chaikoff, Chernick & Felts, 1950) and can be restored by refeeding—particularly with diets rich in carbohydrate. All these findings suggested that a high rate of glycolysis[1] was essential for lipogenesis (for review see Chaikoff, 1953).

This view-point was further investigated by Gurin and his co-workers, who studied the conditions necessary to repair the depleted lipogenic function of liver preparations from diabetic animals (Shaw & Gurin, 1953). In this work they used particle-free extracts of liver, which, when taken from normal rats, could incorporate both acetate and pyruvate carbon into fatty acids, but which, when isolated from the livers of diabetic animals, failed to incorporate either. Lipogenesis from pyruvate by extracts of diabetic livers could not be restored by the addition of mitochondria from normal liver homogenates, but could be partially restored by the addition of the supernatant fraction. Since it appeared that the function of the normal supernatant fraction was to provide the enzymes of the EM pathway, intermediates of this pathway were tested in its place. Of these, fructose 1:6-diphosphate was the most effective. These results thus supported the view that an active glucose dissimilation is essential for lipogenesis, and further, that a block in the synthesis of fatty acids, characteristic of diabetes, could be traced to an enzymic defect in the soluble fraction of the liver cell.

Although, as we have seen, insulin in vitro markedly stimulates incorporation of acetate into long-chain fatty acids by normal liver slices, it had no effect on the incorporation of butyric or caproic acids (Brady & Gurin, 1950a); this suggests that the second block in diabetes is located at the level of the conversion of acetyl-coenzyme A to butyryl-coenzyme A. In this context, the findings of Shaw, Dituri & Gurin (1957) are of considerable interest. These workers have shown that, when the supernatant fraction of normal rat-liver homogenates is added to mitochondria isolated from either normal or diabetic livers, this system is capable of incorporating pyruvate carbon into fatty acids, whereas the supernatant fraction from diabetic liver does not confer this power. The requirement for the supernatant fraction could be abolished if butyryl-coenzyme A were added to the medium. From these results it was concluded that the supernatant fraction of diabetic liver homogenates lacked a factor necessary for the conversion of the two-carbon unit to butyryl-coenzyme A. It is of interest to note the considerable difference in the time required to rectify the two defects in lipogenesis in liver tissue that are characteristic of diabetes. As stated earlier, several days' treatment with insulin in vitro is required to overcome the first block, whereas when fructose 1:6-diphosphate is added to cell-free extracts from diabetic rat livers an immediate stimulation of lipogenesis is observed.

[1] In this review the words "glycolysis" or "glycolytic pathway" are taken to mean the Embden-Meyerhof pathway of glucose breakdown.

2. Mammary Gland

The pioneer experiments of Bloch & Kramer (1948), referred to above, led Balmain, French & Folley (1950) to study the effects of insulin added in vitro on the metabolism of mammary gland slices from lactating rats.[2] The use of this tissue was suggested by the earlier observations of Folley & French (1949, 1950) which indicated, on the basis of respiratory studies, that mammary gland slices could effect net fat synthesis from glucose or glucose plus acetate (rat) or acetate alone (ruminants). Insulin was found to increase the already high respiratory quotient (R.Q.) of rat mammary gland slices metabolizing glucose or glucose plus acetate, and to raise not only the oxygen uptake, but also the consumption of acetate and glucose (Balmain *et al.* 1950; Balmain & Folley, 1951). These findings were later confirmed by Hills & Stadie (1952). It was further found that mammary gland slices from lactating ewes which could effect net fatty acid synthesis from acetate as sole substrate, as judged by the considerable acetate uptake with high R.Q., were unaffected by addition of insulin to the medium, although addition of glucose appeared to increase the rate of lipogenesis (Balmain & Folley, 1951). Later, the conclusions drawn from these respiratory studies were fully confirmed by experiments in which isotopically labelled acetate and glucose were used as substrates (Balmain, Folley & Glascock, 1952, 1954). When mammary gland slices from lactating rats were incubated with acetate 1-^{13}C-2-^{3}H and glucose U-^{14}C, addition of insulin to the medium markedly stimulated the incorporation of both acetate carbons and of glucose carbon into the fatty acids of the slices. The hormone had, however, virtually no stimulating effect on the considerable incorporation of acetate carbon, and the negligible incorporation of glucose carbon, into fatty acids of mammary gland slices from the lactating ewe.

The role of the two major pathways of carbohydrate metabolism, the EM and pentose phosphate (PP) pathways, in contributing glucose carbons for fat synthesis in mammary tissue has been studied in a quantitative manner by Abraham, Cady & Chaikoff (1957). These authors studied the incorporation of glucose 1-^{14}C, glucose 3:4-^{14}C and glucose 6-^{14}C into CO_2 and fatty acids by mammary tissue from lactating rats.

[2] See also Folley & McNaught, *Brit. med. Bull.* 1958, **14**, 207.—Ed.

Insulin added to the medium increased the $^{14}CO_2$ derived from glucose 1-^{14}C, had no effect on that derived from glucose 3:4-^{14}C and decreased that coming from glucose 6-^{14}C. It also augmented the yield of ^{14}C fatty acids from all three specifically labelled glucoses; the incorporation of carbons 3 and 4 of glucose into fatty acids is of particular interest, since this could occur only by a route other than the glycolytic pathway. The calculations of these authors indicated that, although insulin increased the rate of fatty acid synthesis in mammary slices, the relative proportions of glucose carbon entering fatty acids by the EM and PP pathways respectively were unchanged. Incidentally, this is in contrast with the observations of Felts, Doell & Chaikoff (1956) on liver slices from alloxan-diabetic rats treated with insulin. Under these conditions the hormone preferentially increased the incorporation into fatty acids of C-1 of glucose as compared with C-6.

3. Adipose Tissue

The earlier work of Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari (1956) on the effect of insulin on fat synthesis in brown adipose tissue, and the more recent work of the Harvard school on the synthesis of fatty acids in the rat epididymal fat pad and the effect of hormones on this process, has focused attention on the advantages of this tissue for in-vitro studies.

Winegrad & Renold (1958a) have investigated the effect of insulin on lipogenesis and on the rate of CO_2 production from glucose U-^{14}C, pyruvate 2-^{14}C and acetate 1-^{14}C in the epididymal fat pad of the rat in vitro. They found that about half the fatty acids synthesized from glucose arose by way of a pathway in which C-1 is lost, and half by a pathway in which it is not. This was true whether or not insulin was present in the medium. They also found that, while glucose alone stimulated the incorporation of acetate carbon into fatty acids by a factor of 2, the addition of insulin as well as glucose increased it by a factor of 10. In further work, Winegrad & Renold (1958b) found that raising the level of glucose in the medium produced the same effects on CO_2 production, fatty acid, glycerol and glycogen formation as did increasing the level of insulin. Moreover, increasing the level of glucose in the medium caused a greater proportional increase in the rate of oxidation of C-1 of glucose than of C-6, and this was also true when the insulin concentration was increased. Finally, it is of interest to note that, in spite of this greater emphasis on the oxidation of C-1 of glucose, increasing the glucose concentration and addition of insulin both stimulated the incorporation into fatty acids of C-1 and C-6 of glucose to the same extent.

From this work the authors drew two conclusions. First, that in adipose tissue at least, the effect of insulin is probably on the transformation of extracellular glucose into intracellular glucose 6-phosphate, this being taken to include effects both on cell permeability and on the enzymic conversion of glucose to the phosphate ester. In this connexion it may be noted that Barrnett & Ball (1959) have observed, in an electron-microscope study, that insulin stimulates pinocytosis in adipose tissue, and they suggest that this leads to changes in the membrane which would tend to make it more permeable to glucose. Second, the contrast between the effect of insulin on the relative oxidation of the C-1 and C-6 of glucose and on the relative incorporation of these same two carbon atoms into fatty acids lends support to the view (see section 4) that the process of lipogenesis is closely linked with the available supply of reduced triphosphopyridine nucleotide (TPNH). Winegrad & Renold (1958a) emphasize that the extreme sensitivity of adipose tissue to insulin, and its great vascularity and bulk, suggest that it may well be a major site, if not the chief site, of insulin action in the intact organism. Their conclusion that the action of insulin is primarily to increase the glucose 6-phosphate pool of the cell may, however, be peculiar to adipose tissue, since, in contrast to these findings, Beloff-Chain, Catanzaro, Chain, Masi, Pocchiari & Rossi (1956) have found that increasing the level of external glucose does not reproduce the effects of insulin on isolated diaphragm either qualitatively or quantitatively.

4. Insulin and the Dependence of Lipogenesis on Carbohydrate Metabolism

In the last few years there have been great advances in our knowledge of the mechanism of fatty acid oxidation and synthesis, and it is now possible to attempt an interpretation and integration of some of the above results in terms of current ideas about the biosynthesis of fatty acids. There is little doubt that carbohydrates supply both carbon atoms and hydrogen for fatty acid synthesis. The synthesis of fatty acids from carbohydrate precursors involves the formation of a hydrogen-rich substance from a relatively hydrogen-poor one, i.e., the process is essentially reductive and endergonic.

There are two principal theories extant which seek to explain the biochemical processes by which acetyl-coenzyme A can be synthesized to long-chain fatty acids. The first of these is essentially a development of the well-known Lynen-Green fatty acid cycle (perhaps better likened to a "spiral"), while the second is a more recent, and, so far, less well worked-out scheme which is currently being developed in Green's laboratory at Madison and elsewhere. In the Lynen-Green scheme (for details, see review by Green (1954)), two reductive steps occur at each turn of the spiral. For the first, the reduction of the β-ketoacyl-coenzyme A to β-hydroxy-acyl-coenzyme A, reduced diphosphopyridine nucleotide (DPNH) is required. The second reductive step, the reduction of the unsaturated acyl-coenzyme A, once believed to require DPNH (with flavine adenine dinucleotide acting as an intermediate carrier), is now, following upon the work of Langdon (1957) on liver tissue, thought to be TPNH-linked. The second, newer scheme (see Brady, 1958) again postulates two reductive steps, but the source of the hydrogens for both of these is believed to be TPNH, although the synthesis will proceed slowly, in pigeon liver, with DPNH as sole hydrogen donor (see Wakil, Titchener & Gibson, 1959). Although lipogenesis may thus be linked (perhaps wholly) to TPNH in liver tissue, this is not necessarily true of other tissues, notably mammary gland, from which a soluble enzyme system, capable of synthesizing short-chain fatty acids with DPNH as sole hydrogen donor, has been isolated (see Hele, 1958). However, it seems likely that in this context the mammary gland is an exception and that, in general, the reduction of the unsaturated acids requires TPNH. Indeed, Hele (1958) has suggested that the mammary gland may have two systems, the first synthesizing short-chain fatty acids, being DPNH-linked, while the second, which forms long-chain fatty acids, may require both DPNH and

TPNH, or perhaps TPNH only.[3] Coming now to the source of the hydrogen for the reductive steps of lipogenesis, there is a certain amount of evidence, which for lack of space cannot be detailed here, that neither the DPNH nor the TPNH generated in the Krebs cycle is available for this process and, if this be the case, then the source of hydrogen used in the reductive synthesis of fatty acids must therefore be localized either in the EM pathway (DPNH) or the PP cycle (TPNH).

The earlier work of the Chaikoff and Gurin schools appeared to suggest that a block in glycolysis was an essential feature of the defective lipogenesis of the diabetic liver. On this view, therefore, lipogenesis was regarded as being specifically linked with the EM pathway of glucose breakdown as a source both of two-carbon units and of hydrogen for reduction. As we have seen, recent work has tended to shift attention away from the glycolytic pathway towards the PP cycle as providing a specific link between carbohydrate breakdown and lipogenesis. This change of emphasis arises from the current concept, just discussed, in which at least one (and perhaps both) of the reductive steps in lipogenesis is thought to be linked to TPNH which, as is well known, is generated by the PP pathway. There is now a growing literature reporting effects of diabetes and insulin on this pathway in relation to lipogenesis. The initial block in carbohydrate metabolism in diabetes, at the level of the conversion of extracellular glucose to intracellular glucose 6-phosphate, would have the effect of decreasing the supply of hydrogen atoms arising from both the EM and the PP pathways, since glucose 6-phosphate is common to both reaction sequences, and hence would diminish fatty acid formation through both routes. Evidence of the effect of diabetes on the activity of the PP pathway is somewhat conflicting, but has recently been summarized by Ashmore & Hastings (1958). They conclude that, although a greater proportion of the sugar oxidized passes by way of the PP pathway in diabetes, the actual amount so oxidized is still very much less than that found in normal animals. As regards the concept of TPNH as a link between carbohydrate breakdown and lipogenesis, Brady, Mamoon & Stadtman (1956) have shown that the addition of either TPNH, or TPNH-generating systems (e.g., TPN plus citrate), to homogenates of liver from diabetic rats, increased the impaired rate of synthesis of fatty acids, whereas the addition of DPN or DPNH (i.e., stimulation or simulation of the EM pathway) had no effect. Turning now to the mammary gland, Abraham & Chaikoff (1959) have provided evidence of close integration of the PP pathway and lipogenesis in this tissue, in that both appear simultaneously on the first day of lactation and disappear together on weaning. It is significant in the present connexion that a precisely similar time relationship was found by Balmain & Folley (1951) for the stimulating effect of insulin in vitro on lipogenesis in rat mammary gland slices. Abraham & Chaikoff (1959) point out that, although there is strong evidence that the link between the PP pathway and lipogenesis is through TPNH, this interpretation presents certain difficulties, since, as first shown for the rabbit by Popják, Folley & French (1949), milk-fat synthesis is known to occur in the mammary gland of the pregnant animal, a tissue in which Abraham & Chaikoff (1959) could find no evidence for the existence of the PP pathway. The uncertainty is heightened by the fact that Abraham, Matthes & Chaikoff (1959) have since reported that TPNH is generated to about the same extent in subcellular fractions from the livers of normal and diabetic rats even though fatty acid synthesis is depressed in diabetes.

These results appear to contra-indicate a connexion between tissue levels of TPNH and lipogenesis, a find of considerable significance if it is confirmed. The results of McLean (1960) on the stimulating effect of the artificial electron acceptor, phenazine methosulphate, on the oxidation of glucose by way of the PP pathway in the mammary glands of pregnant rats can be taken to offer some support for the views of Abraham *et al.* (1959), since the phenazine methosulphate presumably acts in the pregnant gland as does an active fat-synthesizing enzyme system in the lactating gland.

5. Synoptic Discussion

It is clear that, in all three tissues that have been extensively investigated so far, carbohydrate degradation and fatty acid synthesis are closely linked. The consensus of present evidence indicates that, in diabetes, there are two blocks in the linking of carbohydrate metabolism with lipogenesis: the first at the level of the conversion of extracellular glucose to intracellular glucose 6-phosphate, and the second at the level of the conversion of acetyl-coenzyme A to the four-carbon stage. Both major pathways of carbohydrate metabolism appear to be involved, although there is some evidence (see Siperstein, 1959) that the PP pathway may be of greater importance in the insulin stimulation of lipogenesis than is the EM pathway. The question of whether the primary action of insulin is to facilitate glucose utilization and so, by providing TPNH via the PP pathway, secondarily to stimulate fatty acid synthesis, or whether the primary action of insulin is to stimulate fatty acid synthesis and so, by reoxidizing TPNH to TPN, secondarily to stimulate carbohydrate metabolism, is of great interest, and the evidence of Abraham *et al.* (1959) might well turn out to be of considerable significance in this context. The evidence of these authors that the rate of TPNH generation is normal in the livers of diabetic animals, together with the evidence presented earlier that the rate of formation of two-carbon fragments also appears to be undiminished in the diabetic state, would seem to indicate that the second defect in the linking of carbohydrate metabolism with lipogenesis may possibly be an enzymic one, and not one resulting from a shortage of either carbon or hydrogen for lipogenesis.[4]

Observations which are difficult to interpret in the light of the theory that insulin regulates lipogenesis by stimulating the utilization of carbohydrate by the PP pathway concern species differences, already noted, in the effects of insulin on lipogenesis in mammary tissue. As we have seen, mammary slices from ruminants, as exemplified by the sheep, will actively synthesize fatty acids from acetate alone; the incorporation of acetate into fatty acids of the slices is, however, markedly

[3] More recently, Ganguly (1960) has prepared from cow udder tissue an enzyme system which actively synthesizes from malonyl-coenzyme A fatty acids of all chain-lengths from C-4 to C-18. This system corresponds to the second scheme outlined above, a scheme which is linked exclusively to TPNH. An interesting feature of this work is that the amounts of butyric and caproic acids synthesized in relation to the long-chain fatty acids formed resemble the relative proportions found in cow milk-fat. Further, it is possible to calculate from the figures given in this paper that the malonyl-coenzyme A pathway must be a major pathway of fatty acid synthesis in the cow udder.

[4] Since this paper was written, Matthes, Abraham & Chaikoff (1960) have described a specific lesion in the synthesis of fatty acids in the livers of alloxan-diabetic rats. In the liver tissue of alloxan-diabetic rats the TPNH-linked reductase, which reduces crotonyl-coenzyme A to butyryl-coenzyme A, is much diminished in activity. In the microsomes the activity is only 30% that of normal, while in the mitochondria the activity is only 20% of that found with mitochondria from the liver tissue of normal rats.

stimulated by the addition of glucose to the medium, although the entry of glucose carbon into fatty acids is relatively trivial (Balmain & Folley, 1951; Balmain *et al.* 1952, 1954). In ruminant mammary tissue, therefore, like that of the rat, maximal rates of lipogenesis from acetate seem to depend upon the simultaneous utilization of carbohydrate. Moreover, there is evidence that this utilization may not proceed via the EM pathway (see Balmain *et al.* 1954), and may, therefore, follow the PP pathway, for the existence of which in ruminant mammary tissue there is a certain amount of evidence (Wood, Gillespie, Hansen, Wood & Hardenbrook, 1959; McLean, 1960). One would therefore expect that the addition of insulin to the medium should stimulate lipogenesis in ruminant mammary tissue, but, as we have seen, it does not. The reason for these apparent anomalies must remain, for the present, an intriguing enigma.

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INSULIN AND PROTEIN METABOLISM

A. KORNER M.A. Ph.D.

K. L. MANCHESTER M.A. Ph.D.

Department of Biochemistry
University of Cambridge

1 Insulin and protein biosynthesis
  a Experiments with intact animals
  b Experiments with isolated tissues
2 Mechanism of stimulation of protein biosynthesis by insulin
3 Interrelationship of insulin with other hormones in influencing protein metabolism
  a Interrelationship of insulin with growth hormone
  b Interrelationship of insulin with corticosteroids
References

The very striking effects which insulin exercises on carbohydrate metabolism have tended to overshadow its role in controlling the rate of protein biosynthesis. Until recently it has been generally assumed that the influence of insulin on protein metabolism could be explained as secondary consequences of its effects on glucose utilization. In addition, the effects of insulin on protein anabolism have appeared to be of minor quantitative importance compared with the control of this process exerted by pituitary growth hormone. The results of many of the more recent experiments suggest, however, that this view is an over-simplification and that insulin has a more fundamental role in regulating protein biosynthesis.

In this paper we briefly review the evidence that insulin is involved in protein anabolism and then discuss the mechanism of its action on this process. In the last section the interrelationship of insulin with other hormones which influence protein metabolism is considered.

1. Insulin and Protein Biosynthesis

a. Experiments with Intact Animals

The observation that uncontrolled diabetes, whether of natural origin or induced by pancreatectomy or administration of alloxan, is accompanied by loss of body-weight, depletion of tissue protein and a rise in the rate of nitrogen excretion has led to the acceptance of the idea that insulin plays a part in stimulating protein biosynthesis. The excessive nitrogen loss and tissue wastage of the diabetic animal can be prevented by treatment with insulin (Atchley, Loeb, Richards, Benedict & Driscoll, 1933; Chaikoff & Forker, 1950).

Prolonged administration of insulin to normal animals, fed or starved, has not been shown in the majority of cases to have any significant effect on protein deposition or on the nitrogen balance of the animals (Long & Bischoff, 1930; Goldblatt & Ellis, 1931; Ferrill, 1941; Ingle, Evans & Sheppard, 1944; Geiger, Ershoff, Wassermann & El Rawi, 1953). On the other hand, Maassen (1951), cited by Salter & Best (1953), claimed that prolonged treatment of normal rats with insulin did cause growth, and continued treatment with insulin is reported to have small positive effects on the nitrogen balance of normal human beings (Janney & Shapiro, 1926).

Although little direct effect on total protein synthesis can be obtained by treatment of normal animals with insulin, its administration does rapidly lower the amount of amino acids in the blood of several species of animal (Luck, Morrison & Wilbur, 1928), and promotes the uptake of amino acids by skeletal muscle (Forker, Chaikoff, Entenman & Tarver, 1951). Furthermore, the proportions in which ten essential amino acids disappear from the blood of the fasting dog after insulin administration correspond with the relative proportions of these amino acids in the protein of muscle (Lotspeich, 1949). Treatment with insulin also prevents the rise in the blood amino-acid level which otherwise follows evisceration (Mirsky, 1938; Frame & Russell, 1946; Ingle, Prestrud & Nezamis, 1947). These observations suggest that insulin exercises control of protein anabolism in the normal as well as in the diabetic animal. The effects of insulin on blood amino-acid level are, however, transitory and leave little permanent change in the protein balance of the body.

b. Experiments with Isolated Tissues

Convincing evidence that insulin has a direct effect on protein biosynthesis has come from experiments with in-vitro systems in which added insulin stimulates the rate of incorporation of amino acids into protein. Insulin, added in vitro, stimulates the incorporation of radioactive amino acids into the proteins of isolated rat diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958; Wool & Krahl, 1959a), rat epididymal adipose tissue (Krahl, 1959) and rat-heart slices (Wool, personal communication). Krahl (1953) could find no effect of insulin in vitro on amino-acid incorporation into protein of rat-liver slices, although it stimulated the incorporation of labelled glycine into glutathione in this preparation.

A significant rise in amino-acid incorporation by the isolated rat diaphragm can be produced by as little as 0.05 milliunits (2.5 mμg.) of insulin/ml. of medium (Manchester & Young, 1959b), an amount which is well within the physiological range (Randle, 1957). No other hormone studied stimulates amino-acid incorporation into the protein of the isolated rat diaphragm (Manchester & Young, 1959b, 1960a). Incorporation of radioactive amino acid into protein of diaphragm from alloxan-diabetic rats is depressed and is restored to the normal level by treatment of the rat with insulin (Krahl, 1953; Manchester & Young, 1960a).

The results of all experiments in which incorporation of radioactive amino acid into protein is used as a measure of protein biosynthesis must be viewed with some reservation, for it is not certain that incorporation of amino acids can be equated with de-novo synthesis of specific, biologically active proteins. Workers in this field are, however, acutely aware of this problem, and evidence is beginning to accumulate which offers direct or circumstantial support to the assumption, which is implicit in experiments of this type, that in-vitro incorporation of amino acid is a reflection of true protein synthesis (Campbell, Greengard & Kernot, 1958; Bates & Simpson, 1959; Hoagland, 1959).

2. Mechanism of Stimulation of Protein Biosynthesis by Insulin

The idea that the effects of insulin on protein anabolism are entirely secondary to increased glucose uptake by the cell has had to be abandoned in view of the results obtained in experiments with the isolated diaphragm. Manchester & Young (1958) and Wool & Krahl (1959a) showed that the stimulating effect of insulin on amino-acid incorporation into diaphragm protein could be demonstrated in the complete absence of glucose in the medium. Such an effect could still be demonstrated after a preliminary incubation of two hours' duration in a glucose-free medium, a procedure which must have reduced the endogenous glycogen and glucose content of the muscle to very low amounts. The addition of glucose to the medium modified neither the extent of amino-acid incorporation into protein nor the effect of insulin on this process (Manchester & Young, 1958; Manchester, 1960a).

Since it is now well established that insulin influences the movement of sugars into the cells of muscle tissues (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Park & Johnson, 1955; Helmreich & Cori, 1957; Park, Johnson, Wright & Batsel, 1957), it is possible that the stimulating effect of insulin on protein synthesis could arise as a necessary consequence of the stimulation of transport of amino acid into the cell by the hormone. In support of this idea is the observation of Kipnis & Noall (1958) that insulin stimulates the accumulation by isolated diaphragm of the non-utilizable amino acid, α-amino*iso*butyric acid. However, there are several pieces of evidence against this view. Manchester & Young (1960b) could detect no effect of insulin on the accumulation by diaphragm of naturally occurring amino acids. Wool & Krahl (1959b) were able to demonstrate that insulin, added in vitro, stimulated incorporation into protein of amino acids which had been accumulated in the diaphragm before its removal from the rat. Manchester & Krahl (1959) found that insulin stimulated the incorporation into the proteins of isolated diaphragm of ^{14}C from [^{14}C]carboxylic acids and [^{14}C]carbon dioxide. It is reasonable to assume that the amino acids containing ^{14}C that are formed from the [^{14}C]carboxylic acids (Manchester & Young, 1959a; Manchester, 1960b) are synthesized intracellularly, and it is unlikely that insulin influences either the entry of carboxylic acids and carbon dioxide into the cell or the many diverse reactions required for the synthesis of the amino acids from these precursors. It can be concluded, therefore, that at least a part of the effect of insulin on protein synthesis must be exercised on a process more intimately concerned with the sequence of reactions involved in the formation of protein than merely the entry of amino acids into the cell.

In recent years subcellular fractions of rat liver containing microsomes (Zamecnik & Keller, 1954) or mitochondria (McLean, Cohn, Brandt & Simpson, 1958) have been used as experimental systems for the study of protein biosynthesis. These systems have been used to examine the effects of hormones on this process (Korner, 1958, 1959a, 1959b, 1960a, 1960b). The in-vitro incorporation of radioactive amino acids into protein by both mitochondria and microsomes of liver of alloxan-diabetic rats is lower than that of subcellular particles from normal rat liver (Korner, 1960b). Treatment of the diabetic rats with insulin restores towards normal the incorporation of amino acids by the subcellular particles. Treatment of normal rats with insulin enhances the ability of the microsomes from rat liver to incorporate amino acid into protein in vitro (Korner, unpublished work).

Incorporation of amino acids into protein involves several stages. Amino acids enter the cell, or are synthesized inside it, and are activated by specific amino-acid-activating enzymes in the soluble fraction of the cell to form amino-acyl adenosine monophosphate complexes on the surface of the activating enzyme (Hoagland, Keller & Zamecnik, 1956). The activated amino acid now forms a complex with soluble ribonucleic acid present in the cytoplasm of the cell (Hoagland, Zamecnik & Stephenson, 1957), from which it is transferred with the aid of guanosine triphosphate to the microsomal particles of the microsomes (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958), where the assembly of amino acids into polypeptide chains takes place (Littlefield, Keller, Gross & Zamecnik, 1955).

Korner (1960b) found that microsomes from liver of normal rats incorporated almost the same amount of radioactive amino acid into their protein whether they were incubated with soluble fraction from liver of normal rats or from liver of alloxan-diabetic or insulin-treated rats. He concluded that the main effect of insulin on protein synthesis is not exerted on the ability of the enzymes of the cytoplasm to activate amino acids nor on the ability of the soluble ribonucleic acid to form a complex with amino acid, but that insulin does affect the ability of the microsomes to assemble the activated amino acids into polypeptide chains.

It should be emphasized that effects of insulin on this system have been shown only when insulin is administered to the rat before preparation of the subcellular particles. Insulin, added in vitro, has not been shown to affect amino-acid incorporation in these cell-free systems.

3. Interrelationship of Insulin with other Hormones in Influencing Protein Metabolism

It has long been realized that certain hormones other than insulin act in concert with it in controlling protein and carbohydrate metabolism and the effects of diabetes. The excessive nitrogen loss of the fasting diabetic animal can be stemmed by hypophysectomy (Houssay & Biasotti, 1931) and by adrenalectomy (Long & Lukens, 1936), and the interrelationship of insulin with growth hormone and with adrenal corticosteroids has been intensively studied.

a. Interrelationship of Insulin with Growth Hormone

The presence of insulin is required to enable pituitary growth hormone to exert its growth-promoting action in the cat and dog (Young, 1939, 1941, 1944, 1945; Milman, DeMoor & Lukens, 1951; Lukens & McCann, 1955; Gaebler, Liu & Zuchlewski, 1956). Milman *et al.* (1951) showed that treatment of pancreatectomized rats with ox-growth hormone elicited no nitrogen retention unless the animals received more insulin than was normally required to keep them alive and in nitrogen balance. Furthermore, no nitrogen retention was observed in pancreatectomized-hypophysectomized cats treated with growth hormone unless insulin was also given. Somewhat similar results were obtained with the hypophysectomized alloxan-diabetic rat (Best, 1955) and with the rat after pancreatectomy (Scow, 1957). The idea has been put forward that growth hormone exerts its growth-promoting action primarily through a regulation of the action of insulin —that growth hormone promotes insulin secretion and directs

the action of insulin away from carbohydrate metabolism and towards protein biosynthesis (see Ketterer, Randle & Young, 1957). On this hypothesis it should be possible to show that insulin can induce protein anabolism in the absence of growth hormone. This has been done with cats (Lukens & McCann, 1955) and with rats (Salter & Best, 1953; Lawrence, Salter & Best, 1954). The latter workers showed that administration of insulin to hypophysectomized rats produced a gain in body-weight, an increase in width of the epiphysial cartilage and some accumulation of protein. Griffiths (1956) confirmed these observations, but Wagner & Scow (1957) found that insulin caused no increase in the rate of gain of body-weight and protein content that was shown by hypophysectomized rats when they were fed forcibly. The latter workers suggested that administration of insulin had merely increased the food intake of the rats used by Best and his associates and need not necessarily have had a direct effect on protein biosynthesis.

Korner (1960a) has found that treatment of the hypophysectomized rat with insulin enhances the amount of an injected dose of radioactive amino acids that is incorporated into protein by subcellular fractions of rat liver. He has also found that insulin treatment of the hypophysectomized rat enhances the incorporation of amino acids into protein of liver microsomes in vitro. Manchester, Randle & Young (1959) and Kostyo (1959) have found that insulin, added in vitro, enhances incorporation of amino acids into the protein of isolated diaphragm from hypophysectomized rats. These observations support the view that insulin can influence protein anabolism in the absence of growth hormone.

Manchester *et al.* (1959) found that growth hormone depressed the sensitivity of isolated rat diaphragm to the effect of insulin on glucose utilization and, at the same time, slightly increased the sensitivity of diaphragm to the effect of insulin on amino-acid incorporation. A decreased sensitivity of muscle towards the stimulation by insulin of glucose uptake enables the increased level of insulin, secreted under the influence of growth hormone (Ketterer *et al.* 1957; Engel, Albertson, Fredericks & Lopez, 1958), to lead to a higher level of protein biosynthesis without simultaneously producing hypoglycaemia. Apart from the production of extra insulin, growth hormone has a nitrogen-retaining effect realized by increasing the sensitivity of muscle to the effect of insulin on protein biosynthesis. Thus administration of growth hormone to the pancreatectomized rat maintained on a constant dose of insulin results in nitrogen retention (Scow, 1957). However, the hypophysectomized animal is least sensitive to the effect of insulin on protein biosynthesis and it is therefore to be expected that the effect of insulin on growth would be least apparent, and the effects of insulin on carbohydrate and fat metabolism most marked, in such an animal. Growth hormone appears to direct the action of insulin away from carbohydrate and fat metabolism and towards protein synthesis. It is thus not surprising that the growth response of the hypophysectomized rat to treatment with growth hormone differs, both qualitatively and quantitatively, from its response to insulin (Salter, Davidson & Best, 1957; Scow, 1957; Scow, Wagner & Ronov, 1958; Korner, 1960a).

b. Interrelationship of Insulin with Corticosteroids

Long & Lukens (1936) noted that adrenalectomy reduced the nitrogen loss of the diabetic animal. It has been generally accepted that the exaggerated loss of nitrogen shown by the insulin-deficient animal is caused by the secretion of corticosteroids, which is not balanced by the presence of insulin. Corticosteroid administration depresses both the basal level of amino-acid incorporation into protein of isolated rat diaphragm and the effect of insulin on this process (Manchester *et al.* 1959; Wool & Weinshelbaum, 1959).

Administration of insulin to the normal animal and possibly to the hypophysectomized animal stimulates, by its hypoglycaemic effect, the secretion of glucocorticoids. Both of these conditions, hypoglycaemia and increased corticosteroid level, will cause an increased gluconeogenesis and a transfer of amino acids away from extra-hepatic tissues. They will thus tend to nullify the effects of insulin on amino-acid incorporation in extra-hepatic tissue. Growth hormone balances this effect by decreasing the sensitivity of muscle to the effects of insulin on glucose uptake, so avoiding hypoglycaemia and its consequences when more insulin is available. It is for these reasons that the fullest stimulation of protein biosynthesis by insulin is seen only when an increased level of insulin is the result of, or occurs in conjunction with, the presence of an increased level of growth hormone.

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THE MECHANISM OF ACTION OF INSULIN

P. J. RANDLE M.A. Ph.D. M.B. B.Chir.

F. G. YOUNG M.A. D.Sc. F.R.S.

Department of Biochemistry
University of Cambridge

1 Action of insulin on the utilization of glucose
  a The hexokinase theory
  b The permeability theory
  c Influence of insulin on carbohydrate metabolism in isolated adipose tissue
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References

Are the manifold actions of insulin explicable in terms of a single effect on a specific metabolic process, and one which is common to all the different types of cell on which insulin appears to act? Or are the alterations in carbohydrate, protein and fat metabolism which are brought about by insulin in different tissues, in those of muscle and liver for example, due to direct action of the hormone on substantially different metabolic processes? Although no final answer can be given to this question, the outlines at least of the problem it poses have become reasonably clear during the past few years, and the object of this paper is to survey some of the attempts that have been made in recent years to provide a general view of the mechanism of action of insulin.

The diversity of actions of insulin in promoting different metabolic processes, and in stimulating the tissues of one organ to respond in a manner different from those of another, provides no insuperable obstacle to the acceptance of the possibility that insulin may act on a single process. If insulin enhances the utilization of substances by the cellular machinery which is characteristic of the type of cell and of the general conditions, nutritive, hormonal, and so on, which obtain at the time, then the precise metabolic effects of insulin activity may be as diverse as the activities of different types of cell under various conditions are known to be.

1. Action of Insulin on the Utilization of Glucose

a. The Hexokinase Theory

The first attempt to provide a general theory of the action of insulin on the utilization of glucose was made by Carl and Gerty Cori and their collaborators between 1945 and 1950 (Cori, 1946, 1950). According to this view the enzyme hexokinase was a focus of insulin activity, insulin being active in releasing this enzyme from a depressive influence exerted by substances from the pituitary gland and from the adrenal cortex. Both in-vivo and in-vitro evidence in support of this idea was adduced. Since the phosphorylation of glucose under the influence of adenosinetriphosphoric acid and hexokinase is probably an obligatory step in the utilization of glucose by all tissues of the body, an activity of insulin in relieving hexokinase from a depressive influence might explain the effect of insulin on carbohydrate metabolism in general. Nevertheless, since insulin exerts a striking action in a rat from which both the pituitary gland and adrenal glands have been extirpated (Bornstein, 1950), it seemed *a priori* unlikely that the inhibitors from which insulin was believed to relieve hexokinase could emanate only from these glands.

Weil-Malherbe & Bone (1951) observed that plasma from diabetic patients contained material which could inhibit the action of hexokinase from the brain of rats, while in researches concerning the diabetes-inducing action of extracts of ox anterior pituitary tissue Reid, Smith & Young (1948) found that some, though by no means all, of the extracts which were active in this respect could depress hexokinase activity in vitro in a manner which was reversed by the in-vitro addition of insulin. Reid *et al.* (1948) suggested that a transformation might occur in vivo of the diabetes-inducing substance such as to render it capable of reversibly depressing hexokinase activity in vitro. Subsequently Bornstein (1953) prepared from the serum of rats made diabetic by alloxan a lipoprotein fraction which depressed the uptake of glucose by diaphragm from a normal starving rat incubated in vitro in the presence of crystalline ox-serum albumin. The stimulating action of a low concentration of insulin added in vitro upon glucose uptake by the diaphragm was diminished by this lipoprotein fraction. An active lipoprotein fraction could not be prepared from the serum of normal rats or from that of hypophysectomized alloxan-diabetic rats. Bornstein & Park (1953) observed that the injection of pituitary growth hormone or of cortisone separately into diabetic hypophysectomized rats did not induce the appearance in the serum of the inhibitory substance, but that the simultaneous injection of these hormones did so. These results were interpreted by Bornstein and Park in terms of the formation, under the influence of pituitary growth hormone and adrenal steroids, of a serum lipoprotein with inhibitory properties towards glucose uptake by muscle. Bornstein (1953) suggested that such a lipoprotein might act by depressing the activity of hexokinase, and subsequently Krahl & Bornstein (1954) obtained evidence that insulin might neutralize the depressive action of the lipoprotein on hexokinase in vitro.

Ilyin & Titova (1956) have claimed that a β-lipoprotein fraction prepared by ethanol fractionation from the blood of starving rabbits depresses in vitro the action of yeast hexokinase and that this inhibition can be released by the in-vitro addition of insulin (Ilyin & Titova, 1956). Although the plasma of a normal fed rabbit, or the β-lipoprotein fraction from it, did not depress yeast hexokinase in vitro, the addition in vitro of cortisone to such plasma or to its β-lipoprotein fraction endowed it with ability to depress the activity of yeast hexokinase in vitro. Unfortunately these interesting claims of Ilyin and Titova so far lack independent confirmation.

The idea that insulin acts primarily on hexokinase has stimulated both controversy and experiment, but the evidence at present available suggests that this idea is unlikely to be valid in the terms in which it was first put forward (see Fisher, 1960). The action of insulin in stimulating the biosynthesis of fatty acids might be explicable on the basis of such a primary action of this hormone (see Folley & Greenbaum, 1960), but

the influence of insulin on amino-acid metabolism could hardly thus be explained (Korner & Manchester, 1960). But there is no doubt that the idea first put forward by Carl and Gerty Cori has been valuable, even though it is not now widely accepted.

b. The Permeability Theory

The introduction by Gemmill (1940) of the use of the excised, isolated rat diaphragm, as a tissue in which the action of insulin added in vitro could be examined, opened a most fruitful field for the study of the action of insulin on isolated tissues. The addition of insulin to rat diaphragm in vitro under suitable conditions can increase the utilization of glucose by over 300% (Randle & Smith, 1958a, 1958b). The much earlier observation that the addition of insulin to the isolated perfused heart could stimulate the uptake of glucose by that organ (Bodo & Marks, 1927; Cruickshank & Shrivastava, 1930) excited less immediate attention, possibly because the glycogen content of the heart of a diabetic animal is abnormally high (Fisher & Lackey, 1925) and the glycogen-storing mechanism in the heart therefore appeared to be unimpaired by insulin deficiency. More recently Bleehen & Fisher (1954) have used the isolated perfused rat heart for the study of insulin action, while Park and his collaborators at Nashville, Tennessee, have also made extensive use of the perfused rat heart in this respect (see below).

When insulin acts on isolated diaphragm some of the glucose which disappears goes not only to glycogen but to other saccharides such as maltose, maltotriose, and other similar substances (Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari, 1955; Beloff-Chain, Catanzaro, Chain, Masi, Pocchiari & Rossi, 1955). Lactic acid production is also increased (cf. Shaw & Stadie, 1959), while carbon dioxide production may be slightly raised, although there is no consistency in this (Villee & Hastings, 1949). There appears to be no substantial effect of insulin on the oxygen uptake of the isolated diaphragm. The formation of glycogen may involve the intermediate formation of uridine diphosphoglucose (Leloir & Cardini, 1957), and an in-vitro effect of insulin on this pathway has been claimed (Larner, Villar Palasi & Richman, 1959; Villar-Palasi & Larner, 1960). All these effects of insulin are capable of interpretation in terms of a primary effect of the hormone on hexokinase, with subsequent utilization of the phosphorylated glucose by the available metabolic pathways. The effects of insulin on the heart could also be similarly interpreted.

Nevertheless, even before insulin had been isolated, Hober (1914) suggested that the metabolic disturbance in diabetes might result from a depression of the rate at which glucose can enter the cells of the body. Later Loewi (Haüsler & Loewi, 1925) described an action of insulin in stimulating the penetration of glucose into red blood cells, but this idea was not based on incontrovertible experimental evidence and was not immediately fruitful. Pollak & Fehér (1936) used galactose to test the idea that insulin acts by increasing the permeability of tissues and found that, in the rat, the injection of insulin resulted in a significantly greater intracellular concentration of galactose in heart and diaphragm than was seen in the absence of injected insulin. Unfortunately the idea that this might represent a fundamental action of insulin was not followed up.

Soskin & Levine (1937) drew attention to the fact that the rate of uptake of glucose by the extra-hepatic tissues of the dog depends upon the height of the blood-sugar level. This was true for the depancreatized animal, in which there was a lack of insulin, for the normal animal, and also for the insulin-treated normal animal. In the depancreatized animal the tissues failed to take up a significant amount of glucose unless the blood-sugar level was very high; nevertheless a significant rise in the blood-sugar level could compensate for the lack of insulin. Soskin & Levine (1940) concluded that insulin is not essential for the utilization of glucose but that insulin facilitates the entry of this sugar into the metabolic machinery of the cell in an undefined manner.

This idea was developed by Levine and his colleagues (Levine, Goldstein, Klein & Huddlestun, 1949; Levine & Goldstein, 1955) as the result of a study of the effect of insulin on the amount of certain non-utilizable materials in the blood of the eviscerated nephrectomized dog. In particular they used galactose because this monosaccharide is believed not to be metabolized by muscle, and a similar belief was held for other substances which were used, such as sucrose. Their findings showed clearly that after galactose had been given in such a dose as to maintain an almost constant blood-galactose level in the eviscerated nephrectomized dog, the administration of insulin lowered the amount of galactose in the blood. Assuming that galactose was not utilized by the muscles, Levine and Goldstein concluded that insulin had induced this sugar to enter the muscle cells, that is, that insulin had made the cells permeable to galactose. With substances such as sucrose or urea no effect of the administration of insulin was seen and the conclusion was drawn that the action of insulin might well be specific for certain monosaccharides, in which category it seemed reasonable to include glucose. It was therefore inferred that insulin acted on glucose uptake by facilitating the entry of the sugar into the muscle cells in the same way that it facilitated the entry of galactose. Quite independently Ross (1952) obtained direct evidence that insulin increases the rate of entry of glucose into the aqueous humour of the eye in rabbits. Conversely the permeability constant for the entry of glucose from the blood plasma into the aqueous humour was diminished by the establishment of alloxan diabetes. Similarly Ross (1953) found that insulin increases the rate of entry of glucose into rabbit lens in vitro.

The essential findings of Levine and his colleagues have been amply confirmed and extended by the subsequent researches of others. In particular Park and his colleagues, working with the eviscerated rat and particularly with the perfused isolated rat heart, have demonstrated an accelerating action of insulin on the movement of non-utilizable sugars (both hexoses and pentoses) into the interior of muscle cells (Park, 1953, 1955; Park & Johnson, 1955). Furthermore in experiments with the perfused isolated rat heart Park, Reinwein, Henderson, Cadenas & Morgan (1959) have shown that insulin also accelerates the movement of the non-utilizable sugar L-arabinose from intracellular to extracellular water. Findings which lead to similar conclusions concerning the action of insulin have been recorded by Fisher and his colleagues (Bleehen & Fisher, 1954).

The results of experiments with non-utilizable sugars are relevant to the metabolism of glucose only if the movement of these sugars across the cell membrane takes place by a mechanism similar to that by which glucose enters the cell. Since competition for entry into the muscle cells of the isolated rat diaphragm or into the perfused isolated rat heart can be demonstrated between glucose and many non-utilizable

sugars whose entry is accelerated by insulin, the conclusion has been drawn that glucose and these non-utilizable sugars cross the cell membrane by the same mechanism (Battaglia & Randle, 1959; Park *et al.* 1959). Direct evidence for an effect of insulin on the movement of glucose into muscle cells (as opposed to an effect of the hormone on the metabolism of the sugar within the cells) has been obtained by investigating the effect of insulin on the accumulation of free glucose inside the cell. In the eviscerated and in the intact rat, as in the perfused isolated rat heart in vitro, the administration of insulin leads to an accumulation of free glucose within the muscle cell when the concentration of glucose in the extracellular water may be regarded as within the physiological range (Park, 1955; Park & Johnson, 1955; Morgan, Randle & Regen, 1959). With isolated rat diaphragm, the action of insulin can also lead to the accumulation of intracellular free glucose when the external glucose concentration is very high and when the experiments are carried out at 12° C. (Park, Bornstein & Post, 1955). This suggests that insulin is most unlikely to be acting on hexokinase under these conditions, since any stimulation of the action of this enzyme should diminish and certainly not increase the amount of free glucose within the cell. On the other hand these findings are clearly consistent with the view that under these conditions insulin accelerates the entry of glucose into the muscle cell to such an extent that the amount of glucose made available for phosphorylation exceeds the ability of hexokinase to phosphorylate it.

Randle & Smith (1958a, 1958b) have shown that anoxia and cell poisons, which prevent oxidative phosphorylation, all accelerate the movement of glucose and of xylose into isolated diaphragm, while Morgan *et al.* (1959) have obtained similar results with the perfused isolated rat heart. Randle & Smith (1958a, 1958b) suggested that the transport of glucose into the cell is normally inhibited by the availability of energy-rich phosphate substances, one possibility being that adenosine triphosphate (ATP), or some other energy-rich substance, reacts with the carrier to render it inactive. Thus anoxia, and substances which inhibit oxidative phosphorylation, might accelerate the transport by lowering the intracellular level of ATP or other energy-rich phosphate compounds. The action of insulin could be interpreted in terms of inhibiting the phosphorylation of the carrier or of accelerating its dephosphorylation (Randle & Smith, 1960). More direct evidence for this idea is at present lacking and, as Randle and Smith have emphasized, other interpretations of their results are possible at the present time.

Bhattacharya (1959b) finds that the insulin-sensitivity of isolated rat diaphragm is greatly influenced by the ionic composition of the supporting medium, magnesium being especially significant, and concludes that ". . . the insulin-sensitivity of the rat diaphragm is possibly directly controlled by the activity of some magnesium-adenosine triphosphate-linked enzyme in the system" (Bhattacharya, 1959b).

Chain and his colleagues (see Chain, 1959) reject the view that insulin acts by facilitating the entry of glucose into the muscle cell, pointing out that in their experiments the promotion of entry of glucose by raising the concentration of this sugar in the external medium ". . . does not produce the same effect on the pattern of glucose metabolism in the rat diaphragm and in the adipose tissue as does insulin" (Chain, 1959; cf., however, Jeanrenaud & Renold, 1959). But the possibility is not ruled out that glucose may enter the cell by two independent routes, one involving transport, which is stimulated by insulin, the other involving simple diffusion, which is not influenced by insulin (see Bhattacharya, 1959a). This may be particularly the case in the cut isolated rat diaphragm where channels for diffusion into the muscle cell are created during excision of the muscle. The topographical arrangement with respect to the enzymic machinery of the cell of the portals involved in transport might be different from that of those areas of the cell into which glucose may pass by simple diffusion and, if insulin promoted entry only by the portals tied to transport, the metabolic pattern resulting from the action of the hormone might differ from that which followed an enhanced entry by diffusion. Further evidence is needed about the nature of the permeability barriers in cells before any final conclusion can be drawn on this point.

The action of insulin on lipogenesis could be through a primary effect on the movement of glucose across the cell membrane, since this could satisfy the dependence of lipogenesis on the availability of hydrogen from reduced coenzymes (see Folley & Greenbaum, 1960). But this mechanism would not provide a simple explanation for the differences in the action of insulin on different tissues, for example the liver, adipose tissue, mammary gland (Folley & Greenbaum, 1960), although it is not incompatible with it for the reasons discussed above. How far such a process could explain any action of insulin on carbohydrate metabolism in the liver (Fisher, 1960) is by no means clear, but again no immutable incompatibility appears to exist.

c. Influence of Insulin on Carbohydrate Metabolism in Isolated Adipose Tissue

An important difference between muscle and adipose tissues is that muscle lacks the hexose monophosphate oxidative pathway which is a prominent feature of the metabolic activities of adipose tissue. The investigations of Winegrad & Renold (1958) with glucose labelled with ^{14}C in different positions revealed that in isolated adipose tissue insulin increases the formation of carbon dioxide from the C-1 of the glucose molecule more than from the C-6 of this molecule, although lipogenesis from C-1 and C-6 is stimulated to the same extent. An important oxidative pathway for glucose in adipose tissue is by the hexose monophosphate oxidative pathway; but again the extent of the action of insulin on this process could clearly be determined by the enzymic pattern of the tissue, and not by the point of action of insulin. Barrnett & Ball (1959) and Ball & Barrnett (1960) have observed the phenomenon of pinocytosis in isolated rat adipose tissue under the influence of insulin and suggest that this agrees with the view that insulin acts on cell permeability. As far as the action of insulin on glucose utilization in adipose tissue is concerned, therefore, there is no reason to suppose that the primary action of insulin need be essentially different from that on muscle.

d. Influence of Insulin on Carbohydrate Metabolism in the Liver

This subject has already been discussed by Fisher (1960) in the present number of the Bulletin. Ashmore, Hastings, Nesbett & Renold (1956) find that although insulin acts rapidly on the blood-sugar level, the impairment of metabolic processes in the liver of the diabetic animal is only slowly restored under the influence of insulin, and no effect is discernible less than six hours after the administration of insulin.

This implies that, as with the defect in lipogenesis, the effect of insulin is a secondary one. One possibility to be considered is that insulin depresses the activity of glucose 6-phosphatase in the liver, since this enzymic activity is much enhanced in the livers of diabetic animals (see Ashmore & Weber, 1959). The difficulty of demonstrating a significant effect of insulin on the carbohydrate metabolism of the isolated perfused liver, and on that of isolated preparations of liver tissue, might therefore arise from the slowness of a secondary effect of insulin on metabolic processes which might, for example, be set in motion by the presentation of greater amounts of glucose to certain intracellular enzyme systems. If this were true the primary effect of insulin on liver tissue could be similar to that on muscle, but firm evidence for this is lacking.

2. Influence of Insulin on Protein Metabolism

This subject is reviewed in the present Bulletin by Korner & Manchester (1960) and it therefore need not be discussed in detail here. It is clear that the former view of Krahl (1956, 1960), according to which the action of insulin in promoting protein biosynthesis in isolated diaphragm from added amino acids was believed to be enhanced by, or even replaceable by, the addition of glucose to the medium, is no longer tenable (Manchester & Young, 1958a, 1958b, 1959, 1960) (see also Wool & Krahl, 1959).

Manchester & Young (1960) found no effect of insulin on the accumulation of naturally occurring amino acids by isolated rat diaphragm, except for a possible but insignificant effect on that of glycine. Furthermore Manchester & Krahl (1959) found that insulin stimulates the incorporation into the proteins of isolated diaphragm of ^{14}C from [^{14}C]carboxylic acids and [^{14}C]bicarbonate added to the medium, and it is reasonable to suppose that the amino acids containing ^{14}C which are formed from [^{14}C]carboxylic acids (Manchester & Young, 1958a; Manchester, 1960) are synthesized intracellularly. Insulin is unlikely to influence either the entry of carboxylic acids and bicarbonate into the cell or the many diverse reactions required for the synthesis of amino acids from these precursors, and it seems very probable that it must, in part at any rate, exert an action on a process more intimately concerned with the sequence of reactions involved in the formation of protein than merely the entry of amino acid into the cell. Insulin is therefore most likely, in this instance, to play an intracellular role which is not obviously dependent on any effect it may have on the entry of substances into the cell.

3. The Basis of Insulin Action

Chain and his colleagues (Chain, Beloff-Chain & Pocchiari, 1956; Chain, 1959) have emphasized that many of the reactions stimulated by insulin are of an energy-requiring synthetic nature, such as glycogen synthesis from glucose and protein synthesis from amino acids in muscle, fat synthesis in the lactating mammary gland, and both glycogen and fat synthesis in adipose tissue. Such endergonic processes must derive their energy from oxidative reactions, and Chain *et al.* (1956) have suggested that insulin may make this available by raising the energy level in susceptible cells. On the other hand it should be emphasized that insulin can exert a striking effect on the uptake of glucose by muscle under anaerobic conditions (Randle & Smith, 1958a; Morgan *et al.* 1959). Chain (1959) further proposes that this may be achieved, at least in part, by making the TPN–TPNH system (triphosphopyridine nucleotide, oxidized or reduced form respectively) more effective for synthesis. The dependence of the synthesis of fatty acids, and the stimulation of this process by insulin, on the availability of hydrogen from reduced coenzymes, is discussed by Folley & Greenbaum (1960). But evidence at present is lacking that such a coenzyme-linked system is primarily concerned in the insulin-stimulated entry of glucose into the muscle cell, nor do we know sufficient about the process of formation of protein from amino acids in tissues to be able to relate such a coenzyme-linked reaction with the influence of insulin on it. But those synthetic processes which involve the removal of the elements of water (for example, glycogen synthesis and peptide band formation) necessarily depend in part on the reductive removal of oxygen and the oxidative removal of hydrogen (cf. Young, 1948). Thus the availability of coenzymes in a suitably oxidized or reduced form might well be of importance here also.

The transport of sugars into muscle is depressed by poisons which are known to react with —SH groups, though such an effect is not large. Nevertheless the transport of the sugar across the cell membrane may involve its combination with an —SH protein (Battaglia & Randle, 1959). Thus if the carrier of sugar across the cell membrane is active only in a reduced form, the production of which depends on the availability of reduced coenzymes, something akin to Conway's "redox pump" (Conway, 1953) might be relevant. In this way the possibility of a single point of action of insulin, involved in the making available of reduced coenzymes for multiple purposes, including the transport of sugars across the cell membrane, might become eligible for consideration. That such a theory might involve a primary action of insulin on a structure within the cell should not at once rule it out, since we should not limit the properties of cell membranes to those known about non-living permeability barriers. The process of pinocytosis may well be relevant in this respect (Ball & Barrnett, 1960).

We just do not know at present whether or not all the many effects of insulin will ultimately be explained in terms of a single action. Until more experimental facts are available, those who attempt to correlate the many actions of insulin in an all-embracing theory based on the influence of this hormone at a metabolic point common to all the processes which it affects must always be in danger of what Samuel Butler described as "the art of drawing sufficient conclusions from insufficient premises". Nevertheless, the glimmerings of a reasoned unity are beginning to be discernible in the fog of uncertainty.

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ENDOCRINE DISORDERS AND INSULIN ACTION

RUSSELL FRASER M.D. F.R.C.P.

Postgraduate Medical School of London

1 How endocrine disorders affect the hypoglycaemic action of insulin
2 Endocrine disorders involving abnormal insulin action
  a "Simple" obese diabetes mellitus
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Lack of insulin action is probably implied by the diabetic syndrome of wasting and polyuria with hyperglycaemia and glycosuria, since this is so universally relieved by insulin; and conversely the opposite state of spontaneous hypoglycaemia may often imply excessive insulin action. This paper will therefore review the incidence in endocrine disorders of diabetes mellitus, of hypoglycaemia and of tendencies to these disorders, and also the use in clinical endocrinology of some tests of insulin action.

Before the discovery of insulin, disordered carbohydrate metabolism had been seen in human adrenal disease (Porges, 1910), and in pituitary disease (Cushing, 1912), but these glands were established as sources of important antagonists to insulin by Houssay's observation that hypophysectomy ameliorates experimental diabetes mellitus (Houssay, 1936), by Long's observation of a similar effect of adrenalectomy (Long, Katzin & Fry, 1940), and by the induction by Young of both temporary and permanent (metahypophysial) diabetes with injections of pituitary extract (Young, 1951). Study of insulin overdosage in man (Fletcher & Campbell, 1922) soon led to the recognition of spontaneous hypoglycaemia due to anterior pituitary defect (Jacob, 1923), to adrenal cortical defect (Marañon, 1925), and to hyperinsulinism (Howland, Campbell, Maltby & Robinson, 1929), and more rarely in other diseases. Soon insulin tests were evolved, first to assess the effects of hypophysectomy (Houssay & Magenta, 1924), and to distinguish insulin-sensitive and insulin-insensitive diabetics (Cespai & Zoltan, 1927), and such tests are still in use.

1. How Endocrine Disorders Affect the Hypoglycaemic Action of Insulin

Only passing reference can be made here to the extensive experimental studies by which these influences on insulin action have been defined: intravenous glucose tolerance tests, tracing the disposal of [^{14}C]glucose, responses to adrenaline, and the correlation of blood-sugar changes with those of other metabolites (e.g., de Bodo & Altszuler, 1958). The main hormones antagonistic to this action of insulin are the anterior pituitary growth hormone and cortisol or similar adrenal steroids; lesser effects also come from anterior pituitary prolactin, the thyroid hormone, adrenaline, glucagon, and possibly from the gonad hormones.

These interrelations are usually assessed from the post-absorptive blood-sugar level and its responses, a level which results from the balance between the hepatic glucose output and the body's glucose utilization. These rates of the entry and exit of plasma glucose are altered by these hormones, with varying potency, speed and persistence of action; while the nervous system probably adjusts these regulators to maintain a glucose level adequate for utilization. Thus, injected insulin will affect this glucose turnover directly at first, and then also indirectly by inducing responsive increases in the secretion of its hormonal antagonists.

Insulin tolerance test (see also section 3a). This test consists in following the post-absorptive blood-sugar curve after a standard intravenous dose of insulin. Since many non-endocrine factors, including the nutritional state, may also affect this response to insulin, it is important to give the subject, for the three days before any such carbohydrate test, a standard daily intake of carbohydrate (usually of 300 g./day; Himsworth, 1939), and to check his general health. The effects will also depend on the fasting blood-sugar (FBS) level; indeed, the responses cannot properly be compared if the FBS is abnormal; if the FBS is normal, then the values of the blood sugar after the injection of insulin are best expressed as a percentage of the FBS. The insulin curve has two phases: an initial fall whose speed reflects the subject's sensitivity to insulin (uninfluenced by antagonistic responses only for the first six minutes), and the subsequent return to the fasting level which reflects the subject's "hypoglycaemic responsiveness" or the adequacy of his insulin antagonists. Increased peripheral utilization due to the insulin causes the first phase, and a responsive increase in hepatic glucose output the second (Wall, Steele, de Bodo & Altszuler, 1957). However, the terms insulin "hypersensitivity" or "resistance" are often used loosely to cover both phases of the curve. Assessment of the initial fall is difficult because of both the evanescence of its "pure" phase (Monaco, Anderson & Kaulbach, 1959) and the superimposed normal oscillations in post-absorptive blood sugar.

A hypophysectomized animal shows the greatest experimental insulin hypersensitivity[1] (10–100-fold according to the species), which is demonstrable even with minute doses of insulin. This abnormality does not reach its full extent until six weeks after hypophysectomy (de Bodo & Sinkoff, 1953). While basally such animals have a lowered plasma glucose turnover rate (Steele, Wall, de Bodo & Altszuler, 1956), and a correspondingly low insulin secretion rate, the insulin injected causes not only an excessive increase in glucose utilization but also a small and sluggish subsequent response in hepatic glucose output (Wall *et al.* 1957). These defects depend mainly, but not alone, on growth hormone lack, since cortisone in appropriate replacement dosage corrects but little of the defect (de Bodo & Altszuler, 1958); but growth hormone and cortisone are apparently synergistic in repairing the defects (de Bodo & Sinkoff, 1953). Similar lesser effects may be produced by hypothalamic damage, possibly because

[1] Defined by assessing the increase in insulin dosage which normal animals need to give a similar curve.

of a resulting depression of anterior pituitary function (Porter, 1954; Spirtos, Ingram, Bogdanove & Halmi, 1954). The adrenalectomized animal, whose post-absorptive plasma glucose turnover appears normal (Altszuler, Steele, Wall & de Bodo, 1956), responds to small (0.025 unit/kg. body-weight) doses of insulin nearly normally (de Bodo & Sinkoff, 1953), but is hypersensitive to larger or standard doses (0.1–0.25 unit/kg.), apparently because these induce both an excessive glucose uptake and a slow compensatory hepatic response.

Repeated administration of anterior pituitary or adrenocortical extract is a recognized cause of insulin-resistance which may progress to temporary insulin-resistant diabetes, and be followed by either recovery or permanent insulin-sensitive diabetes. Pure growth hormone is diabetogenic in adrenalectomized dogs (Lockett, Reid & Young, 1953), but is ineffective if given along with phlorrhizin (Lukens & Dohan, 1942). Moderate pancreatic islet damage is seen in the animals with reversible diabetes, and grosser pathological changes in those with irreversible diabetes. Thus, meta-hypophysial diabetes is due to islet damage induced by the hyperglycaemia. Similar actions from cortisol or adreno-corticotropic hormone have been well documented both in animals and in man (Conn, Louis & Wheeler, 1948; Rosenberg, Cleroux, Raben, Payne & Astwood, 1951; Conn, 1958; Thorn, Renold & Cahill, 1959). In man, the acute hyperglycaemic action of cortisol is evident within two hours (Thorn *et al.* 1959), is reproducible in all subjects by an adequate dose, and is also associated with some lowering of renal threshold for glucose (Froesch, Winegrad, Renold & Thorn, 1958). The occasional development of permanent diabetes after repeated dosage with cortisol, since it is seen only in susceptible subjects and resembles "simple" diabetes, is doubtless also the consequence of islet damage in subjects with low islet reserves.

Adrenaline and glucagon have been fairly clearly shown to increase hepatic glucose output, by their activation of liver phosphorylase and so of hepatic glycogenolysis (Sutherland, 1956). However, these emergency mechanisms are apparently an unimportant or inessential part of the normal blood-sugar response to insulin hypoglycaemia (de Bodo & Sinkoff, 1953). Yet rarely over-production of adrenaline, but not of glucagon, has been found as the cause of a diabetic syndrome (see p. 244).

The thyroid hormone also has indirect effects, increasing the rate of monosaccharide absorption, the rate of the body's metabolism, and also its responsiveness to adrenaline. While it increases the glycosuria of animals given phlorrhizin, and raises the FBS of the hypophysectomized dog without, however, ameliorating its insulin hypersensitivity (Soskin, Levine & Heller, 1939), it does not alter the glycosuria of the hypophysectomized-depancreatized dog. Houssay (1944) has shown that, in partially pancreatectomized rats, thyroid extract causes islet hypertrophy followed by atrophy and permanent diabetes mellitus. But in man and other mammals it has less indirect effect on insulin action, for the blood-sugar responses to insulin and the insulin needs of the diabetic are little influenced by the level of thyroid function, provided that the diet is adequate to the thyroid status.

2. Endocrine Disorders Involving Abnormal Insulin Action

a. "Simple" Obese Diabetes Mellitus

By "simple" obese diabetes mellitus is here meant diabetes with obesity but without any other stigmata of endocrine disease. Both the minimal pathological changes that are found in the islets in over half of all cases of simple diabetes (Warren & LeCompte, 1952), and the tendency for simple diabetics to be either of the thin, juvenile, insulin-sensitive type or of the obese, insulin-resistant, adult type, suggest that some other cause involving resistance to insulin is also operative in this second category. But only in the occasional clinically "complicated" diabetic cases mentioned in section 2b has the existence of other endocrine disorders emerged from special studies (e.g., of adrenocortical function: Conn & Fajans, 1956; Mortimer, Irvine, Hopper & Forsham, 1956; Jakobson, 1958; Rifkin, Solomon & Lieberman, 1958). Maclean & Ogilvie (1955), in their estimations of total β-cell mass, found in post-mortems on diabetics a smooth continuum ranging from near zero to normal values. Using a more functional approach, Wrenshall, Bogoch & Ritchie (1952) found that the insulin extractable from pancreases post mortem was nearly zero in juvenile diabetics, and reduced to about 50% in other diabetics. Correspondingly Unger & Madison (1958) found that the response in mild diabetes to a test dose of tolbutamide was subnormal and suggested a defective insulin reserve. Thus there may be a functional defect in the pancreatic islets in all diabetics. However, the great tendency of obesity to precipitate the condition suggests that this state involves some anti-insulin factor (? non-endocrine) which may exhaust the pancreatic reserve and so lead to diabetes; this is suggested by studies in hereditarily obese hyperglycaemic mice in which hypertrophied islets are found post mortem, although in life no blood-sugar response could be elicited to tolbutamide (Gepts, Christophe & Mayer, 1960).

b. "Complicated" Diabetes Mellitus

By "complicated" diabetes mellitus is here meant diabetes which also shows stigmata of other endocrine disease.

i. With Acromegaly

The diabetic syndrome is seen in 20–25% of acromegalics (Cushing, 1912; Natelson, 1954), and occasional glycosuria in up to 40–50%, while the majority show high levels of plasma insulin (Randle, 1956) and also insulin-resistance (Fraser, Joplin & Opie, unpublished work, 1960). The insulin-resistance is not associated with any increase in urinary glucocorticoids (Forbes, Griswold & Albright, 1950). Those subjects with diabetes more often have a diabetic family history than the other acromegalics (21% as against 2%), and usually have developed it 9–10 years after the acromegaly, so that it seems to depend partly on a low islet reserve. The insulin dose needed to control the diabetes varies considerably with different cases and with time, often apparently independently of the activity of the acromegaly (Coggeshall & Root, 1940; Daughady, Perry & MacBryde, 1950).

ii. With Cushing's Syndrome

Again in about 25% of cases this syndrome is associated with manifest diabetes mellitus, which often regresses when the Cushing's syndrome is cured, while 85% show impaired glucose tolerance (Plotz, Knowlton & Regan, 1952; Sprague, 1953); as already noted, it is a much rarer complication of cortisone therapy. This form of diabetes rarely shows ketosis, and fluctuates with feeding, so that fasting may temporarily clear the glycosuria (Sprague, Power & Mason, 1950).

iii. With Anterior Pituitary Deficiency or Adrenocortical Deficiency

Several cases are on record that illustrate each of these combinations of diabetes with deficiency of its major antagonists (deficiency of anterior pituitary—Fraser & Smith, 1941; Mathisen, 1953; Harvey & de Klerk, 1955; Grunberg & Blair, 1957; adrenocortical deficiency—Stanton, Jones & Marble, 1954). In some the diabetes came first and in some the other endocrine disease. Where diabetes is the older disease, a fall in insulin needs is noted (ranging between one-half and one-tenth), which is partly counteracted when cortisone therapy is started. The fall is most marked with pituitary disease, which may lead to an apparent remission in the diabetes (Cheng, Jahraus & Traut, 1953), and more often to troublesome insulin hypoglycaemias. In the first reported case of combined diabetes and pituitary disease, ketotic coma had been complicated by pituitary infarction (Kotte & Vonderahe, 1940); in several others the pituitary deficiency could also be attributed to infarction of the gland (Brennan, Malone & Weaver, 1956; Calvert & Caplin, 1957), which had probably also similarly arisen as a complication of the diabetes.

In another case (Poulsen, 1953), it was reported that moderately advanced diabetic retinitis remitted almost completely some years after pituitary infarction; this startling observation has led to hypophysectomy's being tried as a treatment for diabetic vascular complications (Luft, Olivecrona, Ikkos, Kornerup & Ljunggren, 1955). In these cases, which are probably due to the cerebral vascular disease, the operation was hazardous, involving a 7% mortality and some further risk of cerebral damage, but arrest of the disease has been claimed, although the treatment has not yet been adequately assessed.

iv. With other Endocrine Disease

Rarely insulin-sensitive diabetes mellitus is found as part of a syndrome due to a phaeochromocytoma and is corrected after the latter's removal, but these cases are usually recognizable by the associated evidence of hyperadrenalism, such as the history of hypertensive attacks and the fundal changes which are evidence of severe hypertension (Goldner, 1947; de Vries, Rachmilewitz & Schumert, 1949). In the paroxysmal attack, 50% of cases with phaeochromocytomata show glycosuria and hyperglycaemia (Goldenberg, Serlin, Edwards & Rapport, 1954). Increased or decreased thyroid function may occur with diabetes mellitus, but rarely does it have much influence on the patient's insulin requirements, though this has been claimed. Associated hyperthyroidism often causes instability of the diabetic control, and a great liability to ketosis after even short fasts.

c. Spontaneous Hypoglycaemia

This disorder can be difficult to recognize when the attacks are infrequent, and then recourse is needed to tests demonstrating an abnormal liability to hypoglycaemia, e.g., a standard insulin tolerance test (Fraser, Albright & Smith, 1941), and/or a 48-hour fast. Causes other than hyperinsulinism will mostly be discerned by the associated stigmata of liver disease, glycogen storage disease, renal glycosuria, or adrenal or pituitary defect, and are indeed the rarer causes. Hence clear evidence of a liability to spontaneous hypoglycaemia without any other clinical stigmata is usually acceptable as adequate proof of hyperinsulinism (Conn & Seltzer, 1955). Functional hyperinsulinism occurs only after meals; this usually distinguishes it from organic hyperinsulinism. The latter may be demonstrable either by showing early insulin-resistance towards intravenous insulin (see section 3a), or by plasma insulin assays. But, especially in children, some rarer unusual causes must be remembered (Steiner & Lawrence, 1953; Cochrane, Payne, Simpkiss & Woolf, 1956). Also retroperitoneal tumours, mostly sarcoma-like but sometimes adrenocortical, may cause hypoglycaemia possibly by involving increased glucose utilization (Scholz, Woolner & Priestley, 1957). An occasional accompaniment of spontaneous hypoglycaemia due to hyperinsulinism is a pituitary tumour with or without obvious acromegaly, in Underdahl's interesting polyglandular syndrome (Moldawer, Nardi & Raker, 1954). This is reminiscent of the effect in puppies of injections of growth hormone (Young, 1944), i.e., hyperplastic islets result, but not diabetes mellitus. In this syndrome are found together functional tumours in multiple endocrine glands, especially often in the pancreatic islets, the parathyroids and the anterior pituitary. While this association has been attributed to a tendency to adenomatosis affecting all endocrine glands, it may well also reflect a physiological linkage—excessive growth hormone stimulating by its hyperglycaemic action the pancreatic islets and, perhaps also by another indirect action, parathyroid activity (Fraser & Harrison, 1960).

d. Prediabetes

This rather ill-defined syndrome, involving a liability to complications of pregnancy, to bearing large babies, to obesity and possibly also to other abnormalities, has been recently defined. It has been discerned in many subjects as long as several decades before the overt or diagnosable diabetes has eventually emerged (Jackson, 1954; Conn, 1958). In this early phase the blood-sugar curve may be slightly abnormal, but even before any such abnormality is evident the new cortisone-load test (see section 3b) can often pick out this syndrome; this suggests that the syndrome is characterized by a subnormal insulin reserve, though the essential defect of the syndrome is not yet clear (Conn, 1958).

3. Related Diagnostic Tests

a. Insulin Tolerance Tests

A standard insulin tolerance test (following the blood sugar for two hours after administration of 0.1 unit insulin/kg. body-weight) has long been fairly widely used for various purposes (Fraser *et al.* 1941), but was mainly used for confirming the presence of suspected hypopituitarism or hyperinsulinism. Simpler, more direct tests are now available for the former purpose, but the test is still useful to detect hyperinsulinism, either in its standard form or in one of the two modified forms available. The modification of the standard test introduced by Engel & Scott (1950) involves the intravenous injection of glucose after 30 minutes in order to minimize the patient's hypoglycaemia. In another test (Monaco *et al.* 1959), a smaller dose of 3 units is given and the effect on blood sugar followed carefully over the first six minutes; a fall would be expected normally but is not found in hyperinsulinism because of its initial or direct insulin-resistance; this is rarely discernible from the standard test in which the condition is characterized in the second half of the test by hypoglycaemic unresponsiveness. The insulin test is also useful for measuring

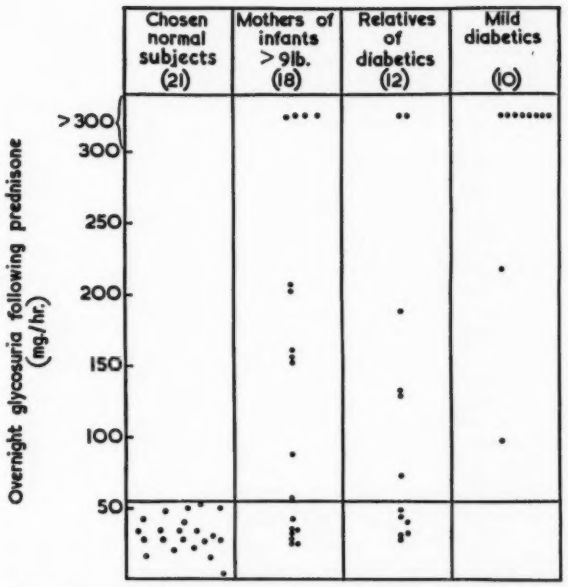
the activity of acromegaly (Fraser, Joplin & Opie, unpublished work, 1960), and for this purpose an augmented dose is used (0.3 unit/kg. or 11.1 units/sq.m.) to reveal the insulin-resistance or hypoglycaemic resistance of this condition. The sum of the blood-sugar values at 60, 90 and 120 minutes (in mg./100 ml.) is normally under 100 in this test; in active acromegaly this value usually lies between 150 and 200.

b. The Cortisone-Load Test for Prediabetes

This test has been the outcome of studies on the hyperglycaemic action of cortisone (Conn, 1958). It occurred to Conn that, since a sufficient dose of cortisone induced hyperglycaemia and glycosuria in normal subjects, a marginal dose might discover potential diabetics. His method has proved, on a five-year follow-up, to be capable of detecting many prediabetics when they give a normal response to glucose tolerance tests (Conn, 1958). An intravenous tolbutamide test (Unger & Madison, 1958) reveals evidence of a low insulin reserve in mild diabetes and may have similar potential uses.

The test, as Conn uses it, comprises two doses of 50 mg. cortisone at eight-and-a-half and two hours before a glucose tolerance test, and is used after a preliminary glucose tolerance test has set aside any subjects that have abnormal findings in glucose tolerance tests. An alternative modification of this test (Joplin & Fraser, unpublished work, 1960) assesses the overnight urinary glucose and blood sugar after the administration of three doses of prednisone during the previous day, i.e., without the oral glucose load (see fig. 1). This test apparently measures the pancreatic insulin reserve or the balance between insulin and its antagonists, which may be clinically abnormal long before overt diabetes mellitus. The occasional instances where severe ketotic diabetes has apparently completely remitted may also still show abnormalities in this test (Stutman & Hayes, 1959).

FIG. 1. Prednisone-Glycosuria Test for Revealing Prediabetes



This chart shows the overnight glycosuria (mg./hr.) induced by three four-hourly doses of 20 mg. prednisone; all subjects tested had no glycosuria on the previous (control) night. (Based on Joplin & Fraser, unpublished work, 1960.)

Note insignificant change induced in normal persons, as compared with the increase in values in mothers of large babies, relatives of diabetics or mild diabetics.

4. Conclusion

Patients with endocrine disease, particularly of the anterior pituitary and less often of the adrenal and other glands, may manifest diabetes mellitus or hypoglycaemia, and such states may be assessed by measuring the blood-sugar response to insulin—a procedure which is particularly useful for assessing spontaneous hypoglycaemia or how active the disease is in patients with acromegaly. Similar tolbutamide response tests can probably measure insulin reserve in mild diabetes, and the more sensitive cortisone-load tests are beginning to define the state of prediabetes.

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INSULIN IN CLINICAL PRACTICE

WILFRID OAKLEY M.D. F.R.C.P.
Diabetic Department
King's College Hospital, London

1 Indications and contra-indications for the use of insulin
2 Scope and limitations of insulin therapy
3 Preparations of insulin and their characteristics
  a Soluble insulin
  b Protamine zinc insulin
  c Insulin zinc suspensions
4 Severe ketosis
5 Reactions to insulin
  a General
  b Local
References

When Banting & Best (1922) published their first full account of the preparation of insulin,[1] it was universally recognized as the greatest advance ever made in the treatment of diabetes mellitus; this view is as true today as it was nearly forty years ago. Since then many efforts have been made to improve on soluble insulin but, except as regards purity and convenience, it is doubtful whether subsequent modifications have added anything really significant to the therapeutic value of Banting and Best's original preparation. In fact something can be said for the view that the introduction of long-acting insulins has only added to convenience at the expense of an increased incidence of the late complications of the disease.

1. Indications and Contra-Indications for the Use of Insulin

The greatest single indication for the use of insulin in the treatment of diabetes is ketosis, and the more severe the ketosis the greater is the indication for giving insulin. In this connexion it is important to distinguish between the ketosis of uncontrolled diabetes and that of carbohydrate starvation, so often seen in the over-dieted but relatively mild diabetic. The greatest single contra-indication to the use of insulin is obesity and, unless there is some good reason such as severe ketosis or active retinopathy, insulin should then be avoided, treatment being aimed at weight reduction by means of a restricted-carbohydrate and low-calorie diet. As a general rule, insulin is essential in the treatment of all children and adolescents, most young adults and a proportion of the aged, while middle-aged and especially obese diabetics tend to respond better to purely dietetic treatment. In the opinion of most authorities, complications such as diabetic cataract in the young, active retinopathy, neuropathy and pregnancy demand the best possible diabetic control and, in most cases, the use of insulin; there is, however, a minority who hold the view that insulin therapy is contra-indicated in diabetic retinopathy, and that it may even favour the development and progression of this complication (Dünner, Ostertag & Thannhauser, 1933; Jahnke, 1959). Occasionally insulin is required to combat pruritus vulvae in an obese diabetic in whom it is impossible by diet alone to control glycosuria; the oral hypoglycaemic agents have proved most valuable in this type of case. In the treatment of diabetes there is, however, a justifiable compromise between continuous insulin therapy and the use of diet only. A significant number of obese subjects, especially women, present with severe symptoms such as pruritus, staphylococcal sepsis and urinary infection; in them a brief period of treatment with insulin greatly accelerates the rate of recovery and should not therefore be withheld. In such cases insulin may be discontinued when urgent symptoms have been relieved.

[1] See Best, p. 179 of this number of the Bulletin.—ED.

2. Scope and Limitations of Insulin Therapy

It is impossible in severe or moderately severe diabetes to obtain a physiological degree of blood-sugar control by means of any of the available insulin preparations; this fact is not only the greatest shortcoming of insulin therapy, but also the commonest cause of so-called inability to stabilize diabetes. The most that can be achieved by means of exogenous insulin is to reduce the blood sugar to within normal limits over the period during which it is exerting its maximum hypoglycaemic action; the duration of normoglycaemia will depend upon the type of insulin and frequency of injection, the patient's sensitivity to insulin, the degree of activity and the amount and arrangement of carbohydrate in the diet. The more frequently the insulin is given the more nearly will the control approximate to the physiological, but there is obviously a limit to the number of injections that can be conveniently given or tolerated. Long-acting insulins were welcomed as a likely means of reducing the number of injections without sacrificing the degree of control of the blood sugar, but the long-term results of their use have not fully justified the optimism felt and expressed when they first became available. The individual response to insulin is perhaps the next most important and variable factor to be considered. High degrees of insulin sensitivity make it impossible, without frequent injections, to obtain a sustained hypoglycaemic response, the dose of insulin tolerated without severe hypoglycaemia being too small to act for more than a relatively short period. In the relatively insulin-insensitive patient the use of a larger dose of insulin produces a more sustained hypoglycaemic response, but the lack of sensitivity also inevitably results in a less controlled rise in the postprandial blood-sugar level. Exercise increases the rate of glucose metabolism and lowers the insulin requirement, but is difficult, especially in young active diabetics, to keep constant from day to day. Much can be done by the arrangement of the diet and the introduction of buffer meals to counteract the fluctuations of the blood-sugar level, but the variable factors already mentioned make it impossible at all closely to reproduce, in any but the mild diabetic, a degree of blood-sugar control comparable with that of the normal subject. This fact has been stressed because it constitutes the greatest limitation in the use of insulin in clinical practice, and may well be the underlying cause of the complications which may occur in spite of all efforts to control diabetes with diet and insulin.

3. Preparations of Insulin and their Characteristics

Insulin preparations may be divided into three categories, according to the length and strength of their action. Soluble insulin (SI) stands alone as the shortest and strongest, protamine zinc insulin (PZI) and crystalline insulin zinc suspension (IZS) (ultralente) are the longest and weakest; while globin, isophane (NPH) and amorphous IZS (semilente) are intermediate in both respects. IZS (lente) is a mixture of ultralente and semilente insulins in the proportion of seven parts to three and combines the actions of its components.

a. Soluble Insulin

This remains the only truly indispensable insulin, and has no substitute in the treatment of severe ketosis and established diabetic coma. When given twice a day, in my opinion it affords a degree of control equal to and usually better than that obtainable by means of any other insulin preparation and, in many parts of the world, there has in recent years been a return to this method of treatment. It is especially valuable in young diabetics from the age of about ten onwards and is superior in respect of reliability and predictability; its chief disadvantages are the necessity for two injections a day and the tendency for some patients, notably the highly insulin-sensitive, to relapse during the three or four hours before the next injection. This latter tendency can to a large extent be overcome by the addition of PZI or isophane insulin to either evening or morning injections or to both. A convenient method is to add 4–12 units of PZI to the evening injection of SI and to give it before afternoon tea (at about 4 p.m.); this shortens the necessary period of action of the morning dose, and prevents early morning relapse (Lawrence & Oakley, 1944).

When relatively large doses of 80 units or more are required for maintenance of control, two injections of SI are preferable, on grounds of both efficiency and safety, to the use of methods of treatment by a single injection of long-acting preparations; this applies equally to combinations of soluble insulin and PZI, and to the IZS. In cases of insulin-resistant diabetes, with a daily requirement of 200 units or more, SI is the only preparation which will control the blood-sugar level, and very large doses of 1,000 units or more may be required to prevent relapse into severe ketosis. In those rare cases in which such insulin-resistance is due to the presence of insulin antibodies, a striking reduction in insulin requirement may be achieved by the oral administration of prednisone. (See Collens & Banowitch, 1955; Colwell & Weiger, 1956; Gitelson & Wislicki, 1956; Oakley, Field, Sowton, Rigby & Cunliffe, 1959.)

b. Protamine Zinc Insulin

This long-acting insulin was first introduced by Hagedorn in 1936 (Hagedorn, Jensen, Krarup & Wodstrup, 1936) and, although its hypoglycaemic action covers a period of 24 hours or more, it soon became apparent that, except in mild diabetics, it failed to control the hyperglycaemia consequent upon the ingestion of carbohydrate, its main action being during the night and early hours of the morning (Lawrence & Archer, 1937; Graham, 1938). To overcome this, PZI may be given at the same time as SI, or mixed with it, in a single morning injection, and this is still a popular method of treatment. The chief drawbacks are failure to control hyperglycaemia following the evening meal, and a certain danger of nocturnal hypoglycaemia. PZI has to some extent been superseded by the IZS (lente insulins) but is still of value in combination with SI, either as a single-injection treatment or as a means of prolonging the action of the latter when this is given twice a day.

Globin insulin was first made available in 1940 but, as its action appears not to differ in any significant way from that of isophane (NPH) insulin, it will not be further considered (Marks, 1940; Duncan & Barnes, 1941).

Isophane (NPH) insulin is a preparation of insulin and protamine in which there is no excess of either component (Hagedorn, 1937, 1938; Peck & Kirtley, 1950; Peck, Kirtley & Ottati, 1952). Moderate doses act for 12–16 hours; large doses may act for 20–24 hours, in which case good control of moderately severe diabetes may be achieved with a single morning injection. As a rule, however, it gives the best results when injected twice a day, either alone or, in more severe cases, in combination with SI.

c. Insulin Zinc Suspensions

These latest additions to the list of insulin preparations were made by Hallas-Møller and his co-workers in Copenhagen (Hallas-Møller, Petersen & Schlichtkrull, 1952). They consist of IZS crystalline (ultralente), IZS amorphous (semilente), and a mixture of the two in the proportion of seven to three, known as IZS (lente). Their prolonged action is essentially due to the use in their preparation of acetate instead of phosphate buffer, as a result of which it is possible to keep the insulin in a state of suspension without the use of protamine or other foreign substance; they also contain small amounts of zinc. IZS amorphous resembles closely isophane and globin insulin in its action, while IZS crystalline is very similar to PZI. Owing to the change in pH neither of these preparations can be mixed with SI without loss of prolonged action. The mixture, IZS, is perhaps the most generally applicable of all insulins for the treatment of diabetes by means of a single daily injection, and its action is comparable with that of a mixture of SI and PZI (Hallas-Møller, Jersild, Petersen & Schlichtkrull, 1952; Gurling, Robertson, Whittaker, Oakley & Lawrence, 1955). It is particularly suitable for very young children and for adults with mild to moderately severe diabetes; after the age of about ten, and in the more severe forms of the disease in adults, the IZS preparations have proved less effective than other methods of treatment referred to above, especially morning and evening injections of SI. This is due to the fact that the action of IZS amorphous is too weak to control the hyperglycaemia which results from ingested carbohydrate, with the inevitable result that heavy glycosuria is liable to occur throughout the day; the danger of nocturnal hypoglycaemia on large doses of IZS is comparable with that attendant on the use of PZI.

Although there is as yet no convincing evidence that single-injection regimes are less efficient in the prevention of late complications in severe diabetics than morning and evening injections of SI, the clinical impression that this may be so is not confined to myself. It is hoped that a survey of a large series of long-term diabetics at present being carried out may throw more light on this important subject.

4. Severe Ketosis

The most urgent clinical use of insulin is in the treatment of diabetic pre-coma and coma, and in these conditions SI should be given at frequent and regular intervals, together with fluids; the disturbance in electrolytes must also be corrected (Nabarro, Spencer & Stowers, 1952). There is some difference in opinion as to the best route of administration of insulin and its optimum dosage. When given intravenously, insulin exerts a rapid action, but this is of short duration, and injections must therefore be made every hour or two hours with frequent blood-sugar control. The intramuscular and subcutaneous routes are slower, but allow of a more prolonged hypoglycaemic action; injections are usually made every three or four hours and less frequent blood-sugar estimations are necessary. The initial dose depends to some extent on whether the patient has previously received insulin and, if so, the amount required, but the initial blood-sugar level is perhaps the best guide to dosage. Young children usually need considerably less than adults, but this is not always the case. Lawrence has suggested

ABNORMAL REACTIONS TO INSULIN INJECTION

(FIGS. A–D)

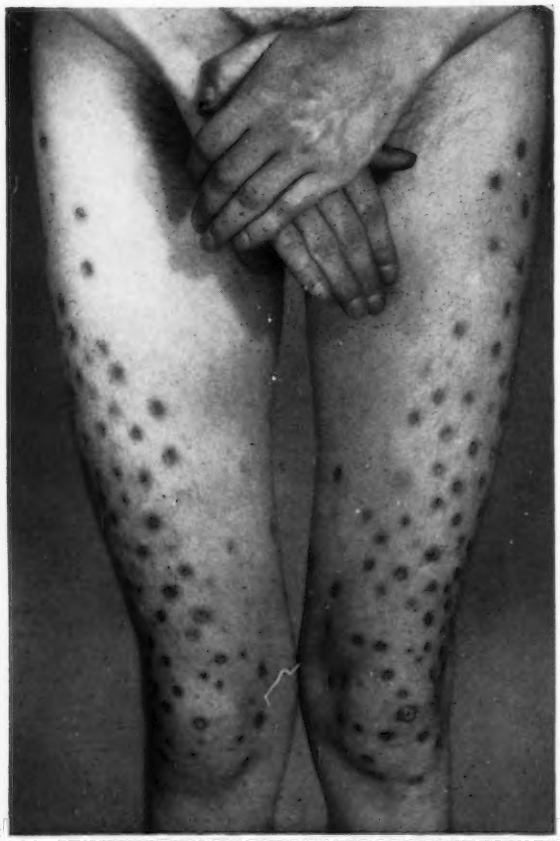
Wilfrid Oakley

(page 247)

A. Symmetrical insulin fat atrophy

B. Insulin tumours

ABNORMAL REACTIONS TO INSULIN INJECTION
(continued)



C. Reaction to intracutaneous injection (early stage)

D. Scarring resulting from intracutaneous injection of insulin (late stage)

that the dose of insulin should be one-tenth of the blood-sugar figure (Lawrence, 1959) and, if this rule is modified for blood-sugar values of 500 mg./100 ml. or less by first subtracting the normal fasting figure of 100 mg./100 ml., the results are most satisfactory. When the initial blood-sugar value exceeds 1,000 mg./100 ml., and especially if the patient has been in coma for many hours, much more insulin may be necessary; in such cases the best guide is the response to the initial dose. In established coma a good method is to give one-quarter of the first injection intravenously and the rest subcutaneously; when there are signs of peripheral circulatory failure, the latter may with advantage be injected into four separate sites in the trunk, rather than into the limbs.

5. Reactions to Insulin

a. General

Hypoglycaemia is the commonest recognizable complication of the use of insulin and, when frequent and severe, must be considered more dangerous than uncontrolled hyperglycaemia. The fault may be with the doctor or the patient; in the former instance common causes are the attempt to render the urine sugar-free after meals, instead of before them, and failure to reduce the stabilizing dose of insulin when a patient leaves hospital for a more active mode of life. The patient may not eat sufficient carbohydrate food at the proper times, or may induce hypoglycaemia by strenuous or prolonged exercise. Prophylactic treatment consists of the use of buffer feeds appropriately timed to suit the particular type of insulin used, and the invariable prescription of a bedtime feed in all diabetics on long-acting insulins.

Recently Kay (1959) has reported good results with cortisone in the treatment of resistant hypoglycaemic coma induced for the treatment of schizophrenia. Glucagon has also been used successfully and, as it can safely be injected by the parents, is particularly useful in the treatment of hypoglycaemic attacks in children. Rarely insulin may produce a generalized allergic response with systemic and cutaneous manifestations, usually urticaria; in addition to the usual immediate measures, it may be necessary to desensitize the patient with frequent small but increasing doses of insulin alone or combined with one of the antihistamine drugs.

b. Local

About ten days after starting treatment with insulin, local reactions, in the form of red painful indurated areas at the site of injection, may occur and persist for two or three weeks, gradually disappearing without treatment. The patient should be warned against the error of regarding them as being due to faulty injection technique or infection.

Insulin atrophy is the most common local reaction to insulin and is much more often seen in women than in men, probably owing to the greater amount of subcutaneous fat in the former. The hollows so produced are unsightly, and in women injections should not be made into the arms or other exposed parts of the body. Insulin atrophy may rarely occur symmetrically in both legs when injections have been given into only one; the distribution may follow that of the cutaneous nerve supply as seen in Plate I, A. The most effective form of treatment at present seems to be repeated injection into the atrophic area.

Insulin tumours are invariably the result of repeated injection into the same area, and are most often seen in children because such tumours become relatively insensitive to the needle. (See Plate I, B.) When large and unsightly they can be removed surgically, but are easily preventable by varying the site of injection.

Intracutaneous injection produces a characteristic picture (Whittaker, 1952), passing through stages of induration, necrosis, ulceration and scarring. (See Plate II, C.) The final picture resembles the scars which may result from a severe attack of smallpox (Plate II, D). The patient usually complains of difficulty in giving the injection and of pain. Some insulin is often lost and the rest poorly absorbed.

Insulin has also been used in clinical practice in the treatment of schizophrenia and of certain wasting conditions, notably those associated with anorexia. Hypoglycaemic shock therapy is reviewed by Sargant & Slater (1947) and has, to a large extent, been replaced by other less hazardous forms of treatment. An interesting feature of this use of insulin is the great variation in dosage required, very large amounts being tolerated by some patients without clinical evidence of hypoglycaemia; the cause of this resistance to insulin is not fully understood.

There are several possible reasons for giving insulin to produce a gain in weight. The most logical is that hypoglycaemia tends to induce hunger and so increase appetite; if this is the object, then the common practice of covering the insulin with glucose is wrong. The anabolic action of insulin may perhaps be regarded as a justification for this form of treatment, but it would seem that insulin is often given empirically for no better scientific reason than that some diabetics on it show a marked tendency to gain weight.

Lastly insulin has been applied locally in the treatment of chronic ulcers, but there is no evidence that this clinical use of insulin is of any value, even in the presence of diabetes.

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CHEMISTRY OF HYPOGLYCAEMIC SUBSTANCES OTHER THAN INSULIN

R. F. MAHLER B.Sc. M.B. M.R.C.P.

Department of Experimental Medicine
Guy's Hospital Medical School, London

1 Salicylates
2 Guanidine derivatives
  a Diguanidines
  b Biguanides
3 Sulphanilyl derivatives
  a Sulphanilyl thiadiazoles
  b Sulphonylureas
4 Hypoglycin A
5 Conclusion
References

Until the discovery of insulin in 1922 (Banting & Best, 1922), many substances were tested for their ability to lower the blood sugar and reduce glycosuria in diabetic patients. Most of the compounds for which such claims had been made were found to be worthless on rigorous examination (von Noorden, 1901). When insulin became generally available, interest fell off in the search for other antidiabetic drugs. A few compounds, notably the synthalins, were given clinical trial, but although they were potent hypoglycaemic agents they were rejected because, in comparison with insulin, they were not as effective in every diabetic patient and they sometimes produced toxic effects.

In recent years, however, a greater appreciation of the complexity of the metabolic problems associated with diabetes has led to a renewed interest in several of these compounds. The accidental discovery of the hypoglycaemic action of carbutamide (Franke & Fuchs, 1955) set off a world-wide search for insulin substitutes which would be effective when given by mouth.

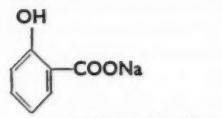
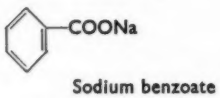
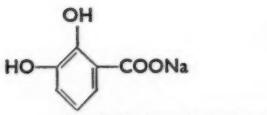
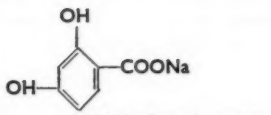
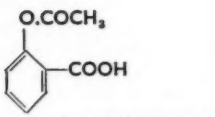
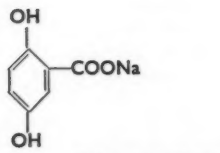
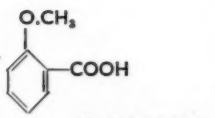
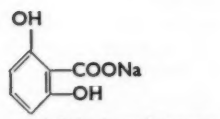
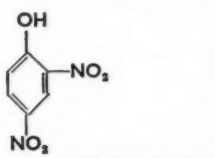
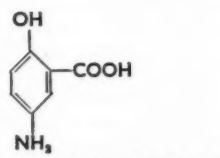
The present paper is limited to a description of compounds of known chemical constitution which have had a critical evaluation of their hypoglycaemic activity.

1. Salicylates

Sodium salicylate and acetylsalicylic acid were among the earliest drugs which effectively reduced the total daily urinary glucose output in diabetic patients (Müller, 1877). Aspirin was used in several clinics but was abandoned with the advent of insulin therapy because of its occasional unpleasant side-effects (Gross & Greenberg, 1948). In 1957 clinical interest in aspirin as a hypoglycaemic agent was again aroused when Reid, MacDougall & Andrews reported an improvement in the state of some of their diabetic patients given fairly large doses of aspirin.

Not all salicylate derivatives possess hypoglycaemic activity. Thus sodium salicylate (Table I, formula I) and acetylsalicylic acid (Table I, formula II) lower the blood sugar, but the closely related sodium gentisate (Table I, formula III) has no effect on the blood-sugar level although it is an effective antirheumatic agent.

TABLE I. Analogues of Salicylate and their Ability to Uncouple Oxidative Phosphorylation

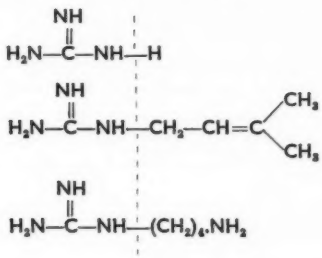
Active substances	Inactive substances
 Sodium salicylate (I)	 Sodium benzoate
 2:3-Dihydroxybenzoate	 2:4-Dihydroxybenzoate
 Acetylsalicylic acid (II)	 Sodium gentisate (III)
 Methyl salicylate	 2:6-Dihydroxybenzoate
 2:4-Dinitrophenol (IV)	 *p*-Aminosalicylic acid (PAS)

Hypoglycaemic activity of the salicylates is in some way related to their ability to inhibit intracellular phosphorylation without interfering with oxidative processes in the cell (Brody, 1956). In this they resemble 2:4-dinitrophenol (Table I, formula IV) which also reduces fasting blood sugar in some diabetic patients and increases glucose uptake by isolated rat diaphragm (Randle & Smith, 1957).

2. Guanidine Derivatives

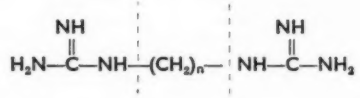
Guanidine (V), which is usually administered in the form of its hydrochloride, is another example of an effective hypoglycaemic agent of the pre-insulin era. Its action on the blood sugar of rats was first described by Watanabe (1918) and subsequently a series of natural and synthetic guanidine derivatives were given a clinical trial in diabetes (Rank, Nothmann & Wagner, 1926).

Galegine (VI), the guanide isolated from the seeds of *Galega officinalis*, was found to have a significant blood-sugar-lowering effect when administered by mouth (Müller, 1925), and agmatine (VII) was found to be the hypoglycaemic agent present in herring sperm. These two compounds were more effective and less toxic than guanidine itself and this observation led to the synthesis of a series of substituted guanides, particularly the diguanidines and the biguanides.

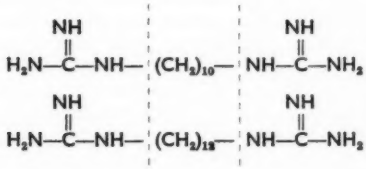


a. Diguanidines

These may be considered as being made up of two guanidine molecules joined by a chain of methylene groups (VIII).



It was found that, within limits, hypoglycaemic activity is directly related to the length of the methylene chain. Of the compounds tested, the most active and relatively least toxic was synthalin A (IX) and synthalin B (X).



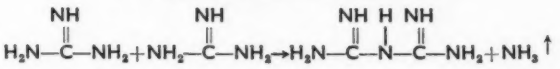
But even these two compounds compared unfavourably with insulin and, when it was thought that they had been responsible for hepatic and renal damage in some patients, their use in the management of diabetes was discontinued.

b. Biguanides

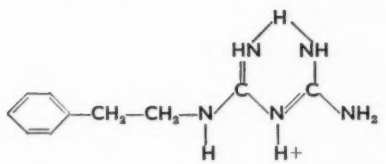
In 1929 Slotta & Tschesche synthesized various biguanides and described their effect on the blood sugar of animals; but these compounds do not seem to have found clinical application at that time. Thirty years later Ungar, Freedman & Shapiro (1957) reported on the hypoglycaemic activity of a newly synthesized biguanide (Table II, formula XVIII), phenformin (DBI), and since then over 200 related biguanides have been tested for their hypoglycaemic activity in animals.

Chemically, the biguanides may be considered to be derived from two moles of guanidine with the elimination of one mole of ammonia (see fig. 1). Each biguanide molecule thus has only five nitrogen atoms in contrast to the six nitrogen atoms of the diguanide molecule.

FIG. 1. Schematic Representation of the Formation of Biguanide from Guanidine

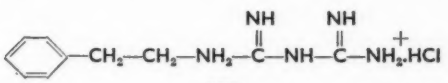


Physical and chemical properties of the DBI molecule lead to the conclusion (Shapiro, 1959) that it is present in vivo in an ionic form, acting as a cation at the pH of tissue fluids, and that the ion assumes the form of an internal six-membered hydrogen-bonded ring (XI). At the pH of gastric juice it is



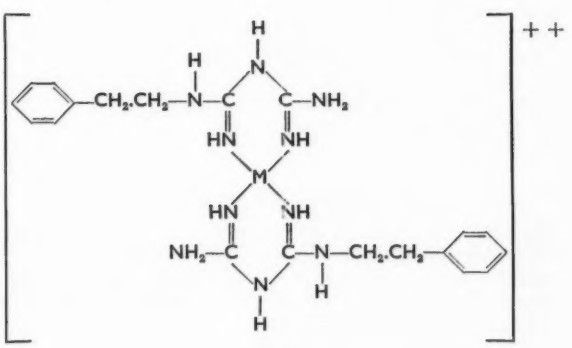
(XI)

present as the dihydrochloride (XII) which is almost certainly a linear structure. Further, biguanides can form a stable



(XII)

chelate ring (XIII), which is thought to reduce the danger of liver d e.

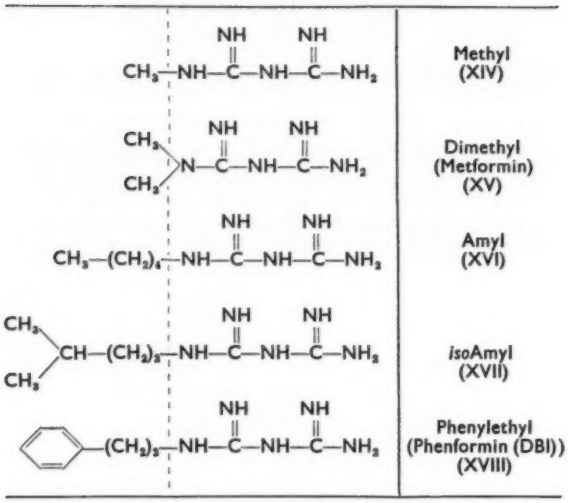


(XIII)

Freedom from toxicity of biguanides is best exemplified by another biguanide, Paludrine (which also possesses slight hypoglycaemic activity), which has been used for years in the treatment of malaria without toxic effects on the liver or kidney.

If more than one amino group on the biguanide molecule is substituted, hypoglycaemic activity is lost. Substitution of one of the hydrogen atoms of the amino group by an alkyl radical gives maximum activity with methyl, the lowest member of the series (Table II, formula XIV), but alkyls containing up to five carbon atoms are also active. Since the introduction of DBI, clinical trials have also been reported of

TABLE II. Biguanide Derivatives



amylbiguanide (Table II, formula XVI) and of *iso*amylbiguanide (Table II, formula XVII) (Odell, Tanner, Steiner & Williams, 1958).

Substitution with longer chains leads to loss of activity. If both the hydrogen atoms of one of the amino groups are substituted, the lowest member of the group, dimethylbiguanide (Table II, formula XV) (metformin) is again the most active compound. This compound was first made by Werner & Bell (1922) and is now being tested clinically (Sterne & Duval, 1959).

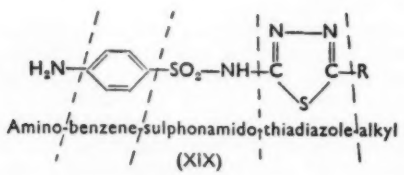
The mechanism of action of this group of compounds is by no means clear. They induce anaerobic glycolysis at unknown sites in the body (Williams & Steiner, 1959) and lower the tissue glucose threshold in diabetic patients (Butterfield, Fry & Holling, 1958).

3. Sulphanilyl Derivatives

These compounds are at present the most widely used insulin substitutes. Several symposia have been held since their introduction in 1955 at which their clinical use, pharmacology and mode of action have been exhaustively, though not conclusively, discussed.

a. Sulphanilyl Thiadiazoles

The first of the sulphanilyl compounds whose hypoglycaemic activity was recognized were the sulphanilyl thiadiazoles (XIX) (Janbon, Chaptal, Vedel & Schaap, 1942).



Loubatières examined the relationship of their structure and their activity in some detail, with the following conclusions (Loubatières, 1944):

i. The presence of the *p*-aminobenzene group is essential for hypoglycaemic activity.

ii. R is an aliphatic side-chain whose structure influences the degree of hypoglycaemic activity. It is maximal for alkyls containing four or five carbon atoms, less for three-carbon alkyls and very much less for alkyls of greater or smaller size (Table III).

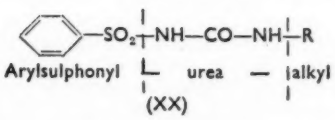
TABLE III. Derivatives of Sulphanilyl Thiadiazole Tested by Loubatières (1944)

R	Name
—CH(CH₃)(CH₃)	*iso*Propyl
—CH₂.CH₂.CH₂.CH₃	*n*-Butyl
—CH₂.CH(CH₃)(CH₃)	*iso*Butyl
—C(CH₃)(CH₃)(CH₃)	Tertiary butyl
—CH₂.CH₂.CH₂.CH₂.CH₃	*n*-Amyl

b. Sulphonylureas

Since the accidental discovery of the blood-sugar-lowering effect of carbutamide (Table IV, formula XXI) (Franke & Fuchs, 1955), many related sulphonylurea derivatives have been synthesized and tested for their effect on the blood sugar (Haack, 1958; Ruschig, Korger, Aumüller, Wagner, Weyer, Bänder & Scholz, 1958; McLamore, Fanelli, P'an & Laubach, 1959).

The arylsulphonylurea molecule (XX) may be considered as a urea derivative with an arylsulphonyl group at one end



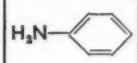
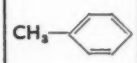
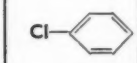
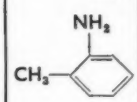
of the urea molecule and another group R, usually an aliphatic side-chain, at the other. The urea group may be substituted by a carbamate or a thiocarbamate, but generally with considerable loss of activity.

To produce the desired effect on the blood sugar, R must be of a certain size and confer lipophilic properties on the whole molecule. In this respect the alkyl substituents give the most potent compounds. The methyl group is inactive, ethyl has some activity, and maximal activity is obtained with straight-chain alkyls containing 3–6 carbon atoms as in tolbutamide (Table IV, formula XXII) and chlorpropamide (Table IV, formula XXIII). Activity diminishes with greater chain-length and is lost completely with alkyls of 12 or more carbon atoms. Aryl substituents at R generally give toxic

compounds, although some, such as metahexamide (Table IV, formula XXIV) are powerful hypoglycaemic agents.

Modifications of the molecule at the benzene ring led to the interesting discovery that, in contrast to Loubatières' compounds, the *p*-amino group is not essential for hypoglycaemic activity of the sulphonylureas, and these new compounds therefore no longer possess antibacterial properties. The first of such compounds to be made was tolbutamide (Maske, 1956), in which the amino group is replaced by a methyl radical. The most potent and longest-acting drugs of this series are the halogen-substituted sulphonylureas, of which chlorpropamide has to date proved clinically the most satisfactory oral antidiabetic agent.

TABLE IV. Sulphonylurea Derivatives

Name	Aryl	R	Hypoglycaemic activity	Duration of persistence of detectable amounts in blood
Carbutamide (XXI)	H_2N— 	C_4H_9	++	33 hours
Tolbutamide (XXII)	CH_3— 	C_4H_9	++	4 hours
Chlorpropamide (XXIII)	Cl— 	C_3H_7	+++	35 hours
Metahexamide (XXIV)			++++	26 hours

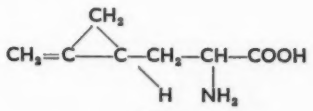
When the amino group is replaced by —OH, —CH_2OH, —COOH, and —NO_2, hypoglycaemic activity is lost entirely.

The various modifications of the molecule affect not only its action on the blood sugar but also the persistence of the drug in an active form in the body and its toxicity. Thus tolbutamide disappears rapidly from the blood through being converted to the inactive carboxy compound (Stowers, Mahler & Hunter, 1958). Chlorpropamide does not appear to undergo metabolic alterations before excretion and persists in the blood for a much longer time (Johnson, Hennes, Driscoll & West, 1959). Carbutamide, possessing a *p*-amino group at the benzene ring, like the antibacterial sulphonamides, also has their toxic effects on the bone-marrow.

In spite of a great deal of work there is still no agreement concerning the site and mode of action of these drugs. It is generally agreed that they reduce hepatic glucose output and act clinically only if endogenous insulin is being produced.

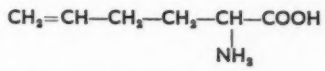
4. Hypoglycin A

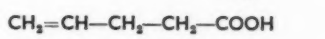
In 1954 Jelliffe & Stuart observed very low blood-sugar levels in Jamaican children suffering from "vomiting disease", due to eating unripe ackee fruit. Hassall, Reyle & Feng (1954) isolated from the fruit two substances which lowered blood sugar in animals, and they named them hypoglycin A and B. The chemical structure of hypoglycin A has been shown to be a new, relatively simple amino acid (XXV) (Ellington, Hassall & Plimmer, 1958); hypoglycin B is the glutamic acid amide of hypoglycin A. Hypoglycin A has now been synthesized (Carbon, Martin & Swett, 1958).

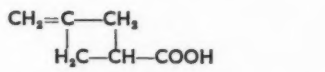


(XXV)

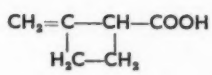
Related compounds with hypoglycaemic activity have been prepared by Anderson, Johnson, Nelson, Olson, Speeter & Vavra (1958) (XXVI, XXVII, XXVIII). Their studies suggest

 2-Amino-5-hexenoic acid (XXVI)

 4-Pentenoic acid (XXVII)

 3-Methylene cyclobutane carboxylic acid (XXVIII)

that neither the cyclopropane ring nor the amino group is necessary for activity, but that the terminal ethylenic linkage CH_2=C— must remain intact, since hydrogenation of the CH_2= radical to CH_3— destroys hypoglycaemic activity. In active compounds the ethylenic radical CH_2=C— is separated by two carbon atoms from the —COOH or the potential carboxyl group which one would expect to be formed in vivo by oxidative deamination and decarboxylation of the amino group of hypoglycin A or of aminohexenoic acid (XXVI). In accord with this suggestion is the absence of hypoglycaemic activity in another synthetic compound (XXIX), in which the ethylenic group is separated by one (or three) carbon atoms from the carboxyl group.

 2-Methylene cyclobutane carboxylic acid (XXIX)

The mode of action of hypoglycin differs entirely from that of insulin (Leppla & von Holt, 1956). Liver glycogen disappears and the blood sugar does not drop until this has happened. The intimate mechanism of this action has not yet been worked out.

5. Conclusion

Recent reviews (Goldner, 1958; Kleiner, 1959; Nabarro, 1959) survey a large number of compounds of known and unknown chemical composition which have been observed under certain conditions to lower the blood-sugar level in man and animals. In this paper an attempt has been made to illustrate the relation between molecular structure and hypoglycaemic activity by the effect on activity of modifications of the molecule. At present it is not possible to discern a unique chemical or physical feature responsible for such action. The search for new types of antidiabetic compounds therefore continues to progress by empiricism and serendipity.

The various antidiabetic compounds and insulin have one thing in common: they reduce the blood-sugar level. The

compounds do not resemble insulin chemically; they do not act like insulin metabolically; and they are neither as effective nor as safe as insulin clinically. But with their help it may be possible to study in greater detail various aspects of the disorders found in diabetes and eventually to define the mode of action of insulin itself.

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CLINICAL EFFECTIVENESS OF INSULIN SUBSTITUTES

J. D. N. NABARRO M.D. F.R.C.P.

Assistant Physician
Middlesex Hospital, London

1 Hypoglycaemic sulphonamide derivatives
  a Selection of patients for treatment with tolbutamide or chlorpropamide
  b Tolbutamide therapy
  c Chlorpropamide therapy
2 The diguanides
3 Comment
References

Patients with diabetes mellitus vary from the thin ketotic "juvenile" type to the obese and stable middle-aged. The former are deficient in insulin and need replacement therapy with insulin for the control of their disease. The latter have some endogenous insulin and often lose their symptoms and hyperglycaemia if weight is lost. Between the two extremes a variable number of patients may be adequately controlled by oral hypoglycaemic agents. Figures from three clinics showing the percentage of patients receiving the different forms of treatment are given in Table I.

TABLE I. Comparative Figures for Different Forms of Treatment of Diabetes Mellitus

Country of clinic	Reference	Patients			
		Total number	Percentage treated by		
			insulin	tablets by mouth	diet alone
Germany 1955	Schöffling, Pfeiffer, Treser, Ditschuneit, Steigerwald & Otto (1957)	498	84	—	16
1957		1,413	44	43	13
USA	Dolger (1959)	1,000	40	40	20
UK	Nabarro (unpublished observations, 1959)	1,184	38	10	52

Duncan (1959) suggests that these percentages should be: insulin, 10%; tablets, 10%; diet, 80%. The differences reflect not only the ideas of the physician on the correct approach to diabetes but also the type of patient attending the clinic. They may explain the diversity of opinion about the proportion of patients who will achieve satisfactory control with oral hypoglycaemic agents. In discussing the clinical effectiveness of insulin substitutes two groups of drugs must be considered, the sulphonamide derivatives and the diguanides.

1. Hypoglycaemic Sulphonamide Derivatives

Clinical experience has shown that these drugs lower the blood sugar only in patients able to secrete insulin. Diabetics who have had a significant degree of ketosis rarely respond to them. The same applies to patients in whom the condition developed before the age of 40, although young diabetics may get transient benefit from their use (Camerini-Davalos, Marble, White, Belmonte & Sargeant, 1957). Four compounds have had extensive clinical trials (formulae I–IV). Two have been discarded on account of toxicity—carbutamide (Kirtley, 1957) and metahexamide (Forsham, 1959); the drugs at present in use are tolbutamide and chlorpropamide.

a. Selection of Patients for Treatment with Tolbutamide or Chlorpropamide

These compounds are of value in the treatment of middle-aged diabetics who do not have ketonuria. Usually these patients are over-weight, and in many cases dietary restriction alone will cure the diabetic symptoms and correct the hyperglycaemia. If the blood sugar remains high after weight reduction or if the patient's weight is within the average range (Kemsley, 1951) when he is first seen, hypoglycaemic sulphonamide derivatives may be used. Long-term treatment of overweight patients with these drugs will relieve diabetic symptoms and lower the blood sugar, but there is usually further increase of weight (Mohnike, Ulrich & Jutzi, 1957), and the dangers of obesity remain. Short courses of treatment have been claimed to produce lasting reduction of blood sugar without preventing weight reduction (Bloom, 1959).

Only a proportion of apparently suitable diabetics respond satisfactorily to tolbutamide or chlorpropamide. The administration of a test dose of 3 g. of tolbutamide and estimation of the blood sugar four hours later has been suggested as a screening method for selecting patients for this treatment (Pfeiffer, Schöffling, Steigerwald, Ditschuneit & Heubel, 1957; Marble, 1958). The four-hour test cannot be used with chlorpropamide (Bradley, 1959). These screening tests do not give trustworthy results (Bradley, 1959), and a therapeutic trial is necessary. Tolbutamide lowers the blood-sugar level by about 100–150 mg./100 ml., and most physicians would agree with the suggestion made by Marble (1958) that, if the blood-sugar level in fasting patients is higher than 250–300 mg./100 ml., a lasting response is unlikely.

Before the introduction of the sulphonamide derivatives a number of patients who would now be thought suitable for this type of treatment were given insulin. The possibility of trying tablets of sulphonamide derivatives instead of insulin now has to be considered. If the patient developed diabetes before the age of 40, was under-weight at the time of diagnosis, or gives a history of diabetic ketosis, transfer to sulphonamide derivatives should not be attempted. If the patient seems to fall into the group described above as suitable for these drugs, a trial may be undertaken. If the insulin dose which the patient is receiving is less than 20 units per day it may be reduced or stopped, but, because some patients have been found to maintain normal blood-sugar levels on stopping insulin, treatment with tablets should not be started until hyperglycaemia has been observed. If the dose is larger than 20 units per day there is a danger that the patient may really be of the insulin-deficient type, and ketosis may follow reduction of insulin dose. It is advisable to admit the patient to hospital for trial in this case, and either the same programme may be followed or an acute ketoacidosis test performed as suggested

by Duncan, Lee & Young (1957). One dose of insulin is omitted and, if ketonuria develops in the next 24 hours, response to sulphonylureas is unlikely. If ketonuria does not develop, the urine is tested four times a day for sugar and treatment with tablets begun when glycosuria and hyperglycaemia occur.

b. Tolbutamide Therapy

The half-life of tolbutamide in the body is short and the substance has to be given in divided doses, two or three per day (Baird & Duncan, 1957), preferably after meals to reduce any possible gastrointestinal disturbance. A suitable initial dose is 0.5 g. three times per day; it is now generally agreed that there is no point in giving more than 3 g. per day. Response may be assessed by relief of diabetic symptoms, reduction of glycosuria and correction of hyperglycaemia. Although most patients respond promptly to tolbutamide there are a few in whom the blood sugar takes some weeks to come down (Walker, Slater, Westlake & Nabarro, 1957). Unless there is obvious deterioration in the patient's condition, a therapeutic trial should continue for 4–6 weeks before failure is accepted. Hypoglycaemia is rare with tolbutamide therapy, although severe attacks have occasionally been recorded (McKendry, Kuwayti & Sagle, 1957; Wieland & McBride, 1959). In tolbutamide-treated diabetics, trauma, infections and surgery often lead to loss of control of the diabetes, and temporary return to insulin treatment is usually required.

Patients who have been over-weight in the past tend to gain weight when given tolbutamide (Mohnike *et al.* 1957; Sugar, 1957). The need for continued dieting must be emphasized to the patient when treatment with tolbutamide is started; increase in diet can be allowed only if the patient is underweight and responding satisfactorily to the drug.

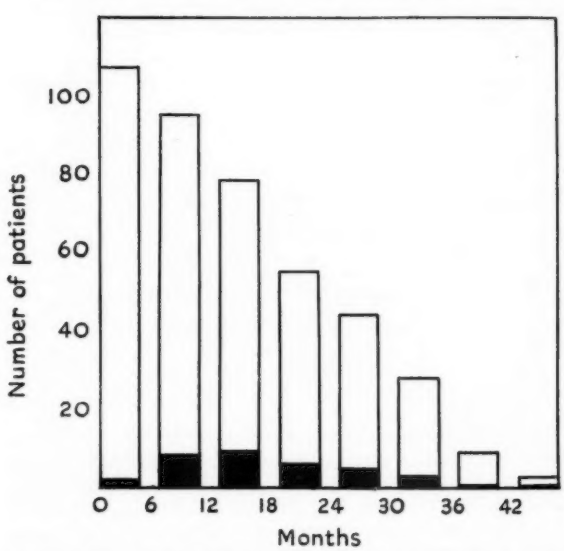
Side-effects of tolbutamide therapy. Experience has shown that side-effects are uncommon with tolbutamide. An irritating urticarial or erythematous rash may occur in 1–2% of the treated patients (Mehnert, Camerini-Davalos & Marble, 1958). Gastrointestinal symptoms are not uncommon; these include epigastric pain and vomiting, and in about 1% of patients they necessitate stopping the drug (Schöffling, Pfeiffer, Steigerwald, Bachrach & Becker, 1957). Exacerbation of duodenal ulceration may occur with perforation (Beaser, 1957) or bleeding (Walker *et al.* 1957; Gelfand, 1959; Stowers, Constable & Hunter, 1959). Haematological abnormalities described include transient leucopenia (Stötter, Seidler, Dorfmüller, Furthmüller & Endres, 1957; Slater, 1959), purpura (Sugar, 1957; Bradley, 1959) and hypoplastic anaemia (Jost, 1959). Serial liver function tests have been performed in many cases but no significant abnormalities have been found. Liver failure has been mentioned in one case but its relation to tolbutamide is uncertain (Bradley, 1959). A case of "toxic nephrosis" attributed to tolbutamide has been described (Schnall & Wiener, 1958). Some patients receiving tolbutamide develop conspicuous facial flushing if they take alcohol (Dolger, 1957). Two minor difficulties may occur: a urinary metabolite of tolbutamide is precipitated by acid and may give a false positive result in the sulphosalicylic acid test for proteinuria (Dolger, 1956); also the prothrombin time may be increased if the patients are already receiving dicoumarol (Chaplin & Cassell, 1958).

Acquired resistance to tolbutamide. This is at present the greatest problem with tolbutamide therapy. Some patients, initially well controlled, after six months or more have a return of glycosuria and hyperglycaemia. In some cases this is due to weight gain or failure to keep to diet, but if the latter is excluded—by admission to hospital and strict supervision—many cases of acquired resistance or secondary failure still remain. The resistance is seldom complete: usually if the tolbutamide is stopped the blood-sugar level rises by a further 100 mg./100 ml. The incidence of acquired resistance to tolbutamide increases with the passage of time—earlier reports gave a figure of about 10% (Pfeiffer, Schöffling, Steigerwald, Treser & Otto, 1957), more recently 30–40% has been reported (Duncan, 1958; DeLawter, Moss, Tyroler & Canary, 1959). Experience in the Middlesex Hospital Diabetic Clinic is illustrated in fig. 1. After the first year, patients still under treatment become resistant at an average rate of 2% per month; this figure may be compared with the 3% per month given by DeLawter *et al.* (1959). It has been noted that patients who respond slowly or indifferently to tolbutamide soon become resistant to it (Walker *et al.* 1957; Marble, 1958).

The mechanism of secondary failure is obscure. It has been found that if the patient was receiving insulin before tolbutamide therapy was started, the insulin requirement is not increased after tolbutamide-resistance has developed (Pfeiffer, Schöffling, Steigerwald, Treser & Otto, 1957; DeLawter *et al.* 1959). Vallance-Owen, Joplin & Fraser (1959) measured the plasma insulin activity in a patient with acquired resistance and found that it failed to increase after a test dose of tolbutamide; later when sensitivity to tolbutamide had been restored by a period of insulin therapy, a test dose produced a rise of plasma insulin activity.

Other uses of tolbutamide. The sulphonylureas produce mitosis of the islet cells in animals (Loubatières, 1957), and the administration of tolbutamide has been advocated as a

FIG. 1. Acquired Resistance to Tolbutamide



The full height of each column represents the number of patients responding satisfactorily at the beginning of the six-month period; the black area indicates the number of patients becoming resistant during the period.

prophylactic measure in early diabetes and prediabetes (Kinsell, Michaels, Brown & Friskey, 1956; Jackson, 1959). It has been claimed to improve control when given with insulin to labile or "brittle" diabetics (Stowers, Mahler & Hunter, 1958; Fabrykant & Ashe, 1959; Volk & Lazarus, 1959). In steroid diabetes the response to tolbutamide therapy is often unsatisfactory (Creutzfeldt & Schlaginweit, 1957; Fajans, Louis, Hennes, Wajchenberg, Johnson, Gittler, Ackerman & Conn, 1957), whereas good results have been reported in diabetes associated with acromegaly (Bergenstal, Lubs, Hallman & Schricker, 1957). In "lipo-atrophic diabetes" response has been poor (Fajans, Louis, Seltzer, Johnson, Gittler, Hennes, Wajchenberg, Ackerman & Conn, 1956; Miller & Craig, 1956). Considerable improvement has been reported in two patients with severe insulin-resistance (Creutzfeldt & Schlaginweit, 1957; Friedlander, 1957).

Hypoglycaemic sulphonamides have been advocated for the treatment of certain types of schizophrenia (Frost, 1958), angina pectoris (Singh & Bardhan, 1959; Fabrykant & Ashe, 1960), and pustular acne (Cohen & Cohen, 1959). An intravenous tolbutamide test has been suggested for the diagnosis of mild diabetes—the hypoglycaemic action develops more slowly in diabetics than in normal persons (Unger & Madison, 1958). This test may also be useful for the diagnosis of insulin-secreting islet cell adenomata of the pancreas. The hypoglycaemic effect may be both greater and of longer duration than in normal subjects (Gittler, Zucker, Eisinger & Stoller, 1958; Fajans & Conn, 1959; Pfeiffer, Pfeiffer, Ditschuneit & Ahn, 1959).

c. Chlorpropamide Therapy

Extensive clinical trials with this sulphonamide derivative started in 1958; experience with it is therefore of shorter duration than that with tolbutamide. The half-life of chlorpropamide in the body is about 35 hours compared with 5 hours for tolbutamide. A single daily dose is sufficient and the blood levels achieved are considerably higher than those of tolbutamide (Knauff, Fajans, Ramirez & Conn, 1959). The maximum effective dose of tolbutamide—3 g. per day—will lower the blood sugar in responsive patients by 100–150 mg./100 ml.: the maximum safe dose of chlorpropamide—0.5 g. per day—has a blood-sugar-lowering effect of 150–250 mg./100 ml. Stowers *et al.* (1959) have published evidence suggesting that this increased potency is only partly due to the higher blood concentration of the drug.

The use of chlorpropamide. This drug may be used as an alternative to tolbutamide in the type of diabetic described in the preceding section. Some patients in whom the response to tolbutamide is inadequate may be controlled with chlorpropamide and it may be effective in some cases of acquired resistance to tolbutamide (Jackson & Oakley, 1959; Sugar, Thomas & Eugenio, 1959; Unger, Madison & Carter, 1959; Herman & Jackson, 1960). The initial dose should be 0.25 g. per day for a frail, elderly patient; and 0.5 g. for a younger, more robust one. Maximum doses may be a little higher but many patients are well controlled on 100–200 mg. per day. The response to treatment is rather slower than with tolbutamide because the maximum blood level is not reached until about the seventh day of treatment (Hamwi, Skillman, Kruger, Roush & Freedy, 1959). Therapeutic trial should if possible be continued for four weeks before being abandoned. Hypoglycaemic attacks are commoner with chlorpropamide than with tolbutamide, and because of slow excretion they may be severe and prolonged (Coates & Robbins, 1959). Chlorpropamide has been used to supplement insulin therapy in patients with labile or brittle diabetes (Dobson, Guilak, Carter, Montgomery & Greene, 1959; Lowenthal, Ross & Tully, 1959).

Side-effects of chlorpropamide. When this drug was introduced the dose recommended—1 g. per day—was, in the light of present knowledge, excessive. Patients treated with this dose often complained of dizziness, drowsiness and confusion; these were not due to hypoglycaemia (Murray, Riddell & Wang, 1958; Walker, 1958). With smaller doses these symptoms are uncommon. Drug rashes occur (Granville-Grossman, Crawfurd, Crowley & Bloom, 1959) and one case of exfoliative dermatitis has been recorded (Brunton, personal communication, 1960). Abdominal symptoms—nausea, vomiting and epigastric pain—are infrequent if big doses are avoided. Transient leucopenia has been reported (Sugar *et al.* 1959), and a few cases of thrombocytopenic purpura (Grace, 1959; Haynes, 1959). Flushing after taking alcohol is more common with chlorpropamide than with the other hypoglycaemic sulphonamide derivatives (Signorelli, 1959).

When chlorpropamide was first used in doses of 1 g. or more per day, it caused obstructive jaundice in a number of patients, with blocking of the bile canaliculi (Brown, Zoidis & Spring, 1959). With the doses at present in use, this complication is rare (Brunton, personal communication, 1960).

Acquired resistance to chlorpropamide. There is no doubt that this occurs, as it does with tolbutamide, but chlorpropamide has not yet been in use for long enough for a valid comparison to be made. The evidence so far suggests that in the period 6–18 months after starting treatment the incidence of acquired resistance to chlorpropamide is about 1% of cases per month (Nabarro, unpublished observations, 1959).

Tolbutamide or chlorpropamide? Initial therapeutic trials with chlorpropamide suggested that its toxicity would be significantly greater than that of tolbutamide. Further experience and adjustment of standard dose-levels have necessitated a revision of this initial impression but a final assessment is not yet possible. There is no doubt that chlorpropamide is more potent as a hypoglycaemic agent, and it is more convenient for the patient to take a single daily dose. On the other hand, the dose has to be adjusted more carefully because of the greater risk of hypoglycaemic attacks.

2. The Diguanides

The diguanides are oral hypoglycaemic agents whose mode of action differs from that of the sulphonamide derivatives. They are less dependent on endogenous insulin and some response may be obtained in insulin-deficient diabetics. The compound most extensively used is phenformin (phenethyldiguanide or DBI); limited trials have been reported with metformin (dimethyldiguanide) (Azerad & Lubetzki, 1959; Granville-Grossman *et al.* 1959). The formulae of these two compounds and of synthalin are shown below (formulae V–VII). The hypoglycaemic diguanides are still in the stage of clinical trial and suggested indications for their use can, at present, only be regarded as tentative.

Indications for the use of the hypoglycaemic diguanides. Patients who appear suitable for treatment with chlorpropamide but fail to respond may be given diguanides either alone or in addition to chlorpropamide (Krall & Bradley, 1959; Pomeranze, Mouraloff, Gadek & King, 1959). Patients with acquired resistance to chlorpropamide may again come under

control if a diguanide is added (Beaser, 1958). Younger diabetics with ketonuria, who would be considered unsuitable for chlorpropamide therapy, may be controlled, at least for a year or so, with phenformin (Krall, White & Bradley, 1958; Walker & Linton, 1959a). There is good evidence that the control of labile diabetes can be improved by the addition of diguanide to insulin therapy: blood-sugar levels are more consistent and hypoglycaemic attacks reduced in number (Bradley, 1959; Lambert, 1959; McKendry, Kuwayti & Rado, 1959; Pomeranze *et al.* 1959).

Technique of diguanide therapy. The diguanides are difficult drugs to use because of the gastrointestinal side-effects. The initial dose should be small—phenformin 25 mg. twice per day, increased at 5–7-day intervals by 25 mg. per day until control is achieved or side-effects prevent further increase. If the patient is already on insulin treatment, the dose should be reduced by 10–20% when the phenformin is started. If response is satisfactory further reduction of insulin dosage will be possible. Metformin has been less extensively tried than phenformin. Larger doses are required, initially 0.5 g. two or three times per day, gradually increasing in some cases to 3.0 g. per day. It has been claimed that this compound is less likely to cause side-effects than phenformin (Azerad & Lubetzki, 1959).

Patients treated with diguanides may have normal blood-sugar levels and marked ketonuria (Hall, Crowley & Bloom, 1958). This may be corrected by increasing the dietary carbohydrate intake, but, if not, insulin treatment should be started. Walker & Linton (1959b) have reported severe acidosis and ketosis with relatively low blood sugar.

Side-effects of diguanides. Gastrointestinal disturbances are a major problem with diguanide therapy. They occur within a few days of starting treatment or increasing the dose. If the initial dose is small there are unlikely to be any symptoms but, if the dose of phenformin has to be increased to 150–200 mg. per day, nausea and vomiting will occur in about half the patients (Marble, 1958). These symptoms are often preceded by decrease of appetite and metallic taste in the mouth (Pomeranze *et al.* 1959). Similar symptoms have been noted with metformin. Diarrhoea may occur with either drug and transient looseness of the bowels is very common in the first ten days of treatment with metformin. Odell, Tanner, Steiner & Williams (1958) have drawn attention to the fact that a number of patients who have been receiving diguanides for several months complain of weakness, lethargy, and loss of weight. To date no serious disturbances of haematopoiesis, liver or kidney function have been reported.

Acquired resistance to diguanides has not been observed in the middle-aged stable diabetics in a two-year period of treatment with these drugs (Pomeranze *et al.* 1959).

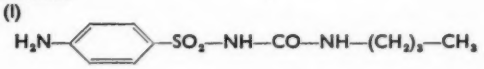
3. Comment

It is too early to attempt any long-term assessment of the place of the sulphonamide derivatives in the treatment of diabetes mellitus. If they act by increasing the pancreatic secretion of insulin, their effect should be physiological. These derivatives are suitable for many elderly diabetics who have high blood-sugar levels but minimal symptoms. Restoring the blood sugar to a normal level is likely to reduce the risk of complications. It is comparatively simple to advise these patients to take tablets to keep the blood sugar within the normal range, whereas it would be difficult to advise a symptomless elderly diabetic to have daily injections of insulin. These tablets may also be of value in patients who find insulin injections difficult on account of physical infirmity or poor vision. On the other hand, tablets should not be given as an alternative to dietetic treatment, and their use in over-weight patients is unjustifiable and should be discouraged.

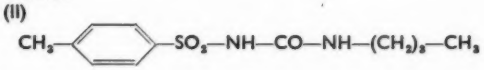
The increasing incidence of secondary failures with tolbutamide and chlorpropamide is a serious problem. If it continues, it seems likely that few diabetics will still be responding satisfactorily after five years' treatment with tolbutamide.

The diguanides must be regarded as pharmacological hypoglycaemic agents without physiological significance. They may reasonably be employed to supplement the sulphonamide derivatives in middle-aged stable diabetics, or insulin therapy in the younger brittle or labile diabetic. It is more difficult to justify their use instead of insulin in the young insulin-deficient diabetic. They may have a temporary action in lowering the blood sugar, and the need for insulin injections may be delayed. Until more is known about the long-term results, it is doubtful whether diguanides should be used—except in carefully conducted clinical trials—to delay insulin injections by a year or two, in a disease that is going to last for the patient's lifetime.

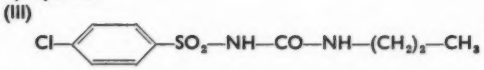
Carbutamide
(I)



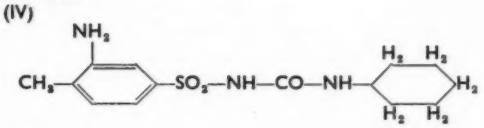
Tolbutamide
(II)



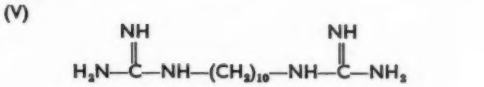
Chlorpropamide
(III)



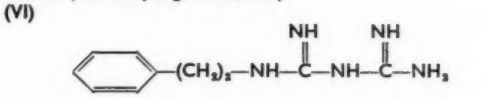
Metahexamide
(IV)



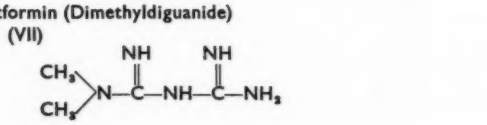
Synthalin
(V)



Phenformin (Phenethyldiguanide, DBI)
(VI)



Metformin (Dimethyldiguanide)
(VII)



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Notes on Contributors

PROFESSOR C. H. BEST is Director of the Banting and Best Department of Medical Research, and Head of the Department of Physiology, University of Toronto. He is also Honorary Consultant to the Connaught Medical Research Laboratories, University of Toronto, Chairman of the Advisory Medical Committee of the Research Defence Board of Canada, and a member of the Scientific Advisory Committee of the Nutrition Foundation. He was the first President of the International Union of Physiological Sciences. In 1921 Professor Best and the late Sir Frederick Banting were the co-discoverers of insulin. He was also the discoverer, with various colleagues, of the enzyme histaminase and of the vitamin choline, and he initiated the work which led to the purification of heparin and its clinical use as an anticoagulant. His published work includes the original paper (with F. G. Banting) on insulin, "The internal secretion of the pancreas" (*J. Lab. clin. Med.* 1922, **7**, 251), and (with D. W. G. Murray, L. B. Jaques & T. S. Perrett) "Heparin and vascular occlusion" (*Canad. med. Ass. J.* 1936, **35**, 621). He is joint author (with N. B. Taylor) of *The physiological basis of medical practice: a text in applied physiology* (6th ed., 1955).

PROFESSOR R. B. FISHER was until last year a university demonstrator in biochemistry in Oxford, and is now Head of the Department of Biochemistry in Edinburgh. His main interests are in protein metabolism and cell permeability. He has worked on creatine synthesis in the perfused heart, on anomalies of the Krebs ornithine cycle mechanism for urea synthesis, on absorption of water and organic solutes from surviving intestine, on gastric hydrochloric acid secretion, and on the glucose metabolism, sugar permeability and oxygen consumption of the perfused heart in relation to the action of insulin. He has also written a monograph, *Protein metabolism* (1954).

DR S. J. FOLLEY is Head of the Physiology Department of the National Institute for Research in Dairying, Shinfield, Reading. In collaboration with Dr Mary L. McNaught he contributed a paper on hormonal effects in vitro on lipogenesis in mammary tissue to a previous number of the Bulletin (*Brit. med. Bull.* 1958, **14**, 207), and in earlier numbers was responsible for a paper on hormones in mammary growth and function (*Brit. med. Bull.* 1955, **11**, 145), and three papers on the physiology of the mammary gland (*Brit. med. Bull.* 1947, **5**, 130, 135 and 142). Dr Folley has recently collaborated with Dr A. T. Cowie in writing a chapter on the mammary gland for the forthcoming 3rd edition of Allen's *Sex and internal secretions*, edited by W. C. Young. At the end of 1959 he was appointed Chairman of the Editorial Board of the *Journal of Endocrinology*.

PROFESSOR RUSSELL FRASER is now Professor of Clinical Endocrinology in the Department of Medicine at the Postgraduate Medical School of London, in which Department he was previously Lecturer and then Reader in Medicine. He graduated from New Zealand University and a few years later started postgraduate work in London on the biochemical and clinical aspects of medicine, first at the Middlesex Hospital and later at the Postgraduate Medical School and the Maudsley Hospital. In 1939 he worked as a Rockefeller Travelling Fellow on endocrine problems with Fuller Albright at the Massachusetts General Hospital, Boston, USA. During the Second World War he worked in psychiatry, first in Mill Hill Emergency Hospital, London, and then on field teams investigating the effects of bombing and the incidence of neurosis in factory workers. After the war he returned to the Postgraduate Medical School and to the clinical endocrinological aspects of general medicine. Professor Fraser's research interests have included thyroid disease, especially the application of radioactive iodine; measurement of the skeletal handling of calcium by tracers; other tests of parathyroid function; the action of sulphonylurea in diabetes; the insulin tolerance test and other tests of pituitary function; methods of pituitary destruction by implantation; the actions of growth hormone in man and its assay in plasma. Publications include: "Radioactive isotopes in medicine" (in: Daley, R. & Miller, H., ed. *Progress in clinical medicine*, 3rd ed., 1956, p. 354); with E. J. King, "Diseases of bone and the parathyroid gland" (in: Thompson, R. H. S. & King, E. J., ed. *Biochemical disorders in human disease*, 1957, p. 352); "Endocrine disorders" (*Annu. Rev. Med.* 1960, **11**, 171).

DR A. L. GREENBAUM is a Reader in Biochemistry at University College, London. During the years 1943–48 he worked with Dr S. J. Folley on studies of the enzymes of the mammary gland and the effect of hormones and lactation on these enzymes. At University College, since 1948, he has continued his work on the mammary gland and has more recently been working on the nucleotide content and turnover in this tissue in relation to lactation. He has also developed the work on the effects of hormones on enzymes in studies of the effect of pituitary growth hormone on fat and protein metabolism. He was invited to the USA in 1954 to lecture on this subject. Present work in this field is related to the effects of the hormone on mitochondrial permeability. His published work, in collaboration with a succession of co-workers, has appeared largely in the *Biochemical Journal*.

DR IEUAN HARRIS is a member of the External Scientific Staff of the Medical Research Council at the Department of Biochemistry of the University of Cambridge. After graduating in chemistry at the University College, Cardiff, he held a Medical Research Council Studentship at the National Institute for Medical Research, London, from 1946 to 1949. In 1949–50 he was James Hudson Brown Memorial Fellow of Yale University School of Medicine, and from 1950 to 1954 a member of the research staff of the Department of Biochemistry of the University of California in Berkeley. During 1954–55 he worked with the late K. Linderstrøm-Lang at the Carlsberg Laboratorium, Copenhagen, and in 1959 was Visiting Research Fellow in the Biological Laboratories of Harvard University. Dr Harris's research work has been mainly concerned with the chemistry of biologically active polypeptides and proteins; in particular, the adrenocorticotropic and melanocyte-stimulating hormones of the pituitary, and the protein constituents of plant viruses and of bacterial ribosomes. His most recent publications have been on the structures of melanocyte-stimulating hormones from the mammalian pituitary (e.g., *Biochem. J.* 1959, **71**, 434, 445, 451; *Nature, Lond.* 1959, **184**, 167).

DR A. J. KENNY is Lecturer in Biochemistry, University of Leeds. He studied medicine in London at King's College and King's College Hospital Medical School. After graduating in 1947 Dr Kenny held several clinical and research appointments in hospitals in Great Britain, Canada and the USA. In 1952 he became Research Fellow and later Instructor in Pharmacology and Experimental Therapeutics at Johns Hopkins University, Baltimore. He returned to England in 1955 and spent three years at Cambridge in the Department of Biochemistry. Dr Kenny's published work includes papers on the incidence of diabetes, the content of extractable glucagon in the human pancreas and the metabolism of glucagon and other peptides.

DR A. KORNER read natural sciences at Cambridge and then carried out research in biochemistry for the Ph.D. degree. He continued to carry out research at the Biochemistry Department in Cambridge as a Mr and Mrs John Jaffé Donation Student of the Royal Society and then spent a year in Berkeley, California, as a Rockefeller Fellow. He was appointed to a university demonstratorship in biochemistry in 1955 and to a lectureship in biochemistry in 1960. He is a member of a Medical Research Council panel which is investigating the effects of human growth hormone in man. Dr Korner has published papers on the biosynthesis of proteins, on the effect of hormones on growth of rats, and on the hormonal control of protein biosynthesis, as well as reviews and articles on growth hormone. He is a Fellow of Clare College, Cambridge.

DR ROBERT MAHLER studied medicine and biochemistry at Edinburgh University. After working at the Postgraduate Medical School of London on various aspects of renal function, he received a grant from the Medical Research Council to study potassium metabolism at the Department of Medicine, University of Manchester. In 1956 he was appointed Lecturer in the Department of Pharmacology and Therapeutics at the University of St Andrews. He spent a year as Eli Lilly Travelling Fellow at the Department of Biological Chemistry at Harvard University Medical School, working on quantitative aspects of carbohydrate metabolism in relation to insulin and other hormones. He is now Lecturer in the Department of Experimental Medicine at Guy's Hospital, London, where he is continuing with his studies on tissue metabolism. His publications include: with S. W. Stanbury, "Potassium-losing renal disease" (*Quart. J. Med.* 1956, n.s. **25**, 21); with W. C. Shoemaker & J. Ashmore, "Hepatic action of insulin: a review" (*Ann. N.Y. Acad. Sci.* 1959, **82**, 452); with R. Schmid, "Chronic progressive myopathy with myoglobinuria: demonstration of a glycogenolytic defect in the muscle" (*J. clin. Invest.* 1959, **38**, 2044).

DR K. L. MANCHESTER is a member of the Department of Biochemistry in the University of Cambridge. He is a Mr and Mrs John Jaffé Donation Student of the Royal Society and a Research Fellow of Sidney Sussex College, Cambridge. He trained as a biochemist in Cambridge, but has also been a Visiting Fellow in the Department of Physiology in the University of Chicago. His work is concerned with the mechanism of the action of hormones, particularly insulin and pituitary growth hormone, in relation to protein metabolism, and he has collaborated in the publication of a number of papers on this subject.

NOTES ON CONTRIBUTORS

DR J. D. N. NABARRO is Assistant Physician to the Middlesex Hospital, London. He was trained at University College Hospital, London, and from 1939 to 1945 he served with the Royal Army Medical Corps. He returned to University College Hospital in 1945 as Medical Registrar and in 1950 was appointed First Assistant to the Medical Professorial Unit. In 1952 he visited the USA as World Health Organization Travelling Fellow, and in 1954 was appointed to the Middlesex Hospital. His main research interests have related to the adrenal cortex and the treatment of diabetes and malignant lymphomata. Publications include: *Biochemical investigations in diagnosis and treatment* (2nd ed., 1958), papers with Drs A. G. Spencer & J. M. Stowers on diabetic ketosis (*Quart. J. Med.* 1952, n.s. **21**, 225; *Lancet*, 1952, **1**, 983), and with Dr G. Walker on Cushing's syndrome (*J. clin. Endocrin.* 1958, **18**, 586). In 1959 he visited Australia as William McIlrath Guest Professor at the Royal Prince Alfred Hospital, Sydney, and in 1960 gave the Oliver-Sharpey Lectures at the Royal College of Physicians in London on the pituitary and adrenal cortex in general medicine.

DR W. G. OAKLEY is Physician in charge of the Diabetic Department at King's College Hospital, London, where he succeeded Dr R. D. Lawrence. After holding the post of Second Assistant to the Medical Professorial Unit at St Bartholomew's Hospital, London, he worked for some years with Dr G. Graham and with him published papers on renal rickets, spontaneous hypoglycaemia and diabetes. Dr Oakley's chief interests since he has been at King's College Hospital have been in diabetes in pregnancy, peripheral arterial disease and insulin-resistance, on which he has published a number of papers in the *Quarterly Journal of Medicine* and current medical journals. Among such publications are the following: with J. Peel, "The management of pregnancy in diabetics" (in: Bourne, A. W. & Nixon, W. C. W., ed. *Transactions of the XIIth British Congress of Obstetrics and Gynaecology, held at Friends House, Euston Road, London, N.W.1, 6th, 7th and 8th July, 1949*, p. 161); "Diabetes in surgery" (*Ann. R. Coll. Surg. Engl.* 1954, **15**, 108); with J. B. Field, G. E. Sowton, B. Rigby & A. C. Cunliffe, "Action of prednisone in insulin-resistant diabetes" (*Brit. med. J.* 1959, **1**, 1601).

DR P. J. RANDLE is a lecturer in biochemistry at the University of Cambridge and an honorary consultant in medical biochemistry to Addenbrooke's Hospital, Cambridge. He studied medicine at Cambridge and University College Hospital Medical School, London, and qualified in 1951. Since 1952 he has been engaged in research on clinical and biochemical aspects of diabetes mellitus and especially on the estimation of insulin and insulin antagonists in blood plasma and on the mechanism of action of insulin and of other factors which regulate carbohydrate metabolism in muscle. He was elected Research Fellow of Sidney Sussex College, Cambridge, in 1954 and since 1957 has been Fellow and Director of Medical Studies at Trinity Hall, Cambridge.

DR F. SANGER, who read natural science at St John's College, Cambridge, has been engaged on research at the Biochemical Laboratory, Cambridge, since 1940; and he has been a member of the external staff of the Medical Research Council since 1951. He studied amino-acid metabolism with Dr A. Neuberger from 1940 to 1943, when he started work on the chemistry of proteins, at first with Dr A. Chibnall, and later independently. Dr Sanger's three most important papers are: "The free amino groups of insulin" (*Biochem. J.* 1945, **39**, 507); with H. Tuppy, "The amino-acid sequence in the phenylalanyl chain of insulin" (*Biochem. J.* 1951, **49**, 463); and, with A. P. Ryle, L. F. Smith & R. Kitai, "The disulphide bonds of insulin" (*Biochem. J.* 1955, **60**, 541). His honours include: a Hutchinson Studentship, 1941–43; a Benn W. Levy Research Studentship, 1942–43; a Beit Memorial Fellowship, 1944–51; a Corday-Morgan Medal and Prize of the Chemical Society, 1951. In 1954 he was elected a Fellow of the Royal Society and a Fellow of King's College, Cambridge. He won the Nobel Prize for Chemistry in 1958, and his paper in this Bulletin is based on the lecture given by him before the Swedish Academy in Stockholm when he was awarded this Prize.

MR G. A. STEWART joined the Wellcome Foundation in 1946 and has been Head of its Biological Control Laboratories since 1949. Most of his work in biological standardization has been concerned with hormones, mainly insulin and posterior pituitary lobe hormones, and with cardiac glycosides. In recent years his interests have extended to a study of orally-active hypoglycaemic agents. Publications include: "An examination of factors affecting the precision of the assay of the oxytocic hormone in posterior pituitary lobe preparations" (*J. Pharm., Lond.* 1949, **1**, 436); "Effets du BZ_{55} et du D_{866} sur certaines activités des hormones du lobe postérieur de l'hypophyse et de l'hexoestrol" (*Ann. Endocr., Paris*, 1957, **18**, 196); and "The actions of digitalis leaf preparations and of cardiac glycosides on the isolated right ventricle of the guinea pig" (*J. Pharm., Lond.* 1958, **10**, 741).

DR K. W. TAYLOR is at present a Research Fellow in Medicine at Harvard Medical School, USA. Before taking up this appointment he was an Elmore Medical Research Student in Cambridge where he completed a Ph.D. in biochemistry. He studied medicine both at Cambridge and at King's College Hospital, London, where he held for a time a junior research post. Always interested in human diabetes, he has published a number of papers relating to the chemical form and mode of action of human insulin and its circulating antagonists. Dr Taylor is to return to the Department of Biochemistry, University of Cambridge, early in 1961.

DR J. VALLANCE-OWEN studied medicine at Cambridge and the London Hospital, where he became a Medical First Assistant. In 1952 he went to the Postgraduate Medical School, Hammersmith Hospital, London, as a medical tutor and senior registrar and began investigating insulin activity and antagonism in plasma; this remains his major research interest. During 1955–56 he worked at the University of Pennsylvania as a Rockefeller Travelling Fellow, in association with Dr Francis Lukens and the late Dr William Stadie. In 1958 he took up his present appointments as Senior Lecturer in Medicine, King's College, University of Durham, and Consultant Physician, Royal Victoria Infirmary, Newcastle upon Tyne. His published work includes: "The histological demonstration of glycogen in necropsy material" (*J. Path. Bact.* 1948, **60**, 325); "Liver glycogen in diabetes mellitus" (*J. clin. Path.* 1952, **5**, 42); and, since 1954, a number of papers on various aspects of insulin activity and antagonism.

DR P. H. WRIGHT is Lecturer in Chemical Pathology at Guy's Hospital Medical School, London. Graduating in chemistry in 1942, he went on to take a medical degree at Liverpool University in 1952 and has been awarded a Rockefeller Travelling Fellowship for the year 1960–61. His early work, written in collaboration with other authors and published in the *Journal of the Chemical Society*, was concerned with the chemical properties of heterocyclic compounds in relation to their trypanocidal properties. For the past five years, after holding various hospital appointments, Dr Wright has concerned himself with insulin-like activity in human plasma, using the isolated rat diaphragm for its detection, and with the properties of serum obtained from guinea-pigs treated with bovine insulin. The results of these latter investigations have been published in the *Lancet*, *Biochemical Journal*, *Journal of Physiology* and *Physiological Reviews*.

PROFESSOR F. G. YOUNG has been Professor of Biochemistry at the University of Cambridge since 1949. He began his career in 1929 as Bayliss-Starling Scholar in biochemistry at University College, London. In 1933 he became Honorary Research Assistant at the Department of Physiology, University of Aberdeen, and in 1934 Research Associate at the Department of Physiology at the University of Toronto, returning in the same year to University College, London, as Assistant in the Department of Physiology, Pharmacology and Biochemistry. He was a member of the Scientific Staff of the Medical Research Council at the National Institute for Medical Research, London, from 1936 to 1942; from 1942 to 1945 was Professor of Biochemistry in the University of London at St Thomas's Hospital Medical School; and from 1945 to 1949 at University College. He was Chairman of the Editorial Board of the *Biochemical Journal* from 1942 to 1946 and is now Chairman of the British National Committee for Biochemistry and of the Medical and Scientific Section of the British Diabetic Association. Professor Young is the author of almost two hundred scientific papers which have appeared in the *Biochemical Journal* and other scientific and medical journals, and he contributed a paper on experimental stimulation of lactation (galactopoiesis) to an earlier number of the Bulletin (*Brit. med. Bull.* 1947, **5**, 155). He was elected Fellow of the Royal Society in 1949.

Book Reviews

The Mechanism of Action of Insulin

Edited by *W. A. Broom, F. W. Wolff & F. G. Young*. Oxford: Blackwell Scientific Publications, 1960. (A symposium organized by the British Insulin Manufacturers: Allen & Hanburys Ltd., Boots Pure Drug Co. Ltd., British Drug Houses Ltd., Burroughs Wellcome & Co. Ltd.) xvi + 320 pages; figures; plates. 23 × 15 cm. £1 12s. 6d.

Meetings for the discussion of current problems in research are a commonplace nowadays and the subsequent publication of their proceedings is frequently undertaken—the expense often being defrayed by the pharmaceutical firms under whose aegis the meeting may have been organized. The Symposium reported in this publication was held in London in September 1958. Although the subject of the discussion was theoretically narrow it was in practice wide, and all the papers were presented and debated by chemists and clinicians with a common interest; they were vigorous, forthright and controversial. Although it is nearly 40 years since the discovery of insulin, there still remains considerable obscurity and much disagreement about its actions. Perhaps it was the discovery of the oral hypoglycaemic agents that rekindled interest in this important subject, which previously had for years occupied the attention of only a limited number of laboratories.

Sessions in this Symposium were devoted to the effect of insulin on cellular transport systems, on intermediary metabolism and on protein synthesis. These subjects, about which considerable controversy exists, were debated by authorities some of whom held widely divergent views, and the same remarks apply to the consideration of the effect, if any, of insulin on the liver. Sessions were also devoted to insulin antagonists, hypoglycaemic agents other than insulin, and the bio-assay of insulin, and these subjects were fully discussed. An important paper was read in which the relation of insulin to the metabolism of adipose tissue was expertly reviewed.

Almost all the experimental results presented at this Symposium had been published before or were published shortly after the meeting. Nevertheless, by gathering all the papers into one volume, the reader is saved much searching through the literature and also has the additional benefit of the inclusion of the penetrating questions and discussions which accompanied them. The material contained in the book is of the highest standard, and the controversial problems are presented and debated by the leading authorities in America and Europe. The publication therefore will be warmly welcomed by all those interested in the subject.

Derrick Dunlop

The Diabetic ABC: A Practical Book for Patients and Nurses

R. D. Lawrence. 12th ed. London: H. K. Lewis & Co. Ltd., 1960. ix + 82 pages. 22 × 14 cm. 5s. 6d.

Home Guide for the Diabetic: Diet Instruction, Recipes, and Other Guidance

Based on *A concise handbook as used in the Leicester Royal Infirmary Diabetic Clinic*. London: Iliffe & Sons Ltd., 1960. 30 pages. 19 × 13 cm. 3s.

The Complete Cookery Book for Diabetics

Iris Holland Rogers. 2nd ed. London: H. K. Lewis & Co. Ltd., 1959. 134 pages; 1 plate; illustrations. 22 × 14 cm. 6s.

It is generally agreed that diabetics should know something about their disease. How much they should know depends on their intelligence and on their mental make-up. Intelligent patients can be taught to undertake most of the management of their disease for themselves, except in emergencies. The unintelligent must have an exact regime laid down for them. To know that the long-term complications are now the diabetic's most serious risk and that they are probably less common in well-controlled diabetics—this may act as a healthy incentive to good control in some patients. But it may reduce others, by nature worriers, to a futile state of anxiety. This illustrates the great difficulty of writing books for diabetics.

One might question Dr Lawrence's categorical statement that "medical science knows why diabetics cannot burn sugar properly", but his ABC is a well-tried guide for patients, informative, highly practical and incorporating the author's well-known "black and red line" method of dieting. For the diabetic bon viveur there is also a table showing the sugar content of different alcoholic drinks. Chablis, which should be the driest of white burgundies, is shown as containing more sugar than white bordeaux; while hock, which is often sweet and occasionally syrupy, is shown as containing no sugar. In general, however, this book can be thoroughly recommended as a practical guide. It presupposes a modicum of intelligence in the reader.

The *Home guide for the diabetic*, based on a concise handbook as used in the Leicester Royal Infirmary, contains much less information and is frankly less good value for money for the average diabetic reader. So far as it goes and as a supplement to instructions given in a clinic, it is good.

Miss Rogers' *Complete cookery book for diabetics* shows that even the strictest diabetic diet need never be dull. It is first-class value and should be in the hands of every diabetic who cooks for himself, or has someone interested to cook for him. It will also be found a useful cookery book by the non-diabetic.

R. R. Bomford

Glutathione

Organized and edited by *E. M. Crook*. Cambridge: University Press, 1959. (Biochemical Society Symposium No. 17.) 116 pages; figures. 25 × 16 cm. Cloth binding: £1 2s. 6d.; paper binding: 15s.

The seven short papers in this volume will be useful to biochemists and physiologists as an up-to-date appendix to the Ridgefield, Connecticut, Symposium,[1] which gives a more extensive account of the subject. The general reader seeking information on a particular point in the biochemistry of glutathione would be well advised to consult the earlier Symposium first, both for authoritative accounts of early work and for incisive discussion of the likely functions of the substance in living material.

Although the present publication does not supersede the Ridgefield Symposium as an authoritative general survey, it is particularly valuable for its reviews of subjects neglected at Ridgefield. Such reviews include that by H. McIlwain on glutathione in nervous tissues and L. W. Mapson's authoritative essay on enzyme systems associated with the oxidation and reduction of glutathione in plant tissues. Work which has appeared in the interval of four years between the two Symposia is adequately documented throughout. There is much new material in S. G. Waley's concise summary of his own work on ophthalmic acid and of the biochemical behaviour of glutathione in the lens. There is also an admirable short account by F. A. Isherwood of the chemical properties of glutathione. C. G. Thomson & H. Martin describe techniques for determining the tripeptide in extracts of animal tissues, P. C. Jocelyn discusses glutathione metabolism in animals and D. B. Hope contributes an account of radiation protection by substances containing sulphur and an interesting discussion of the possible mechanism involved.

Viewed as a whole, the Symposium seems to have lacked the valuable cement of critical public comment (only one contribution to discussion is given). There are some inconsistencies which should have been resolved either by discussion or by stricter editing. For example, the oxidation-reduction potential of the glutathione system at pH 7 is given on p. 33 as −0.40 v, while on p. 86 another contributor discusses and questions a value which corresponds to about −0.32 v. One looks in vain for an elucidation of the important, if difficult, question of what is the true value.

The volume is clearly printed and reasonably priced. It has no index. A small collection of useful papers by British authors, it is a different type of publication from the expensive international conglomerations which burden our consciences by remaining unread on our shelves. As such, it is a worthy addition to the series of Biochemical Society Symposia.

F. A. Holton

[1] Colowick, S., Lazarow, A., Racker, E., Schwarz, D. R., Stadtman, E. & Waelsch, H., ed. (1954) *Glutathione (Proceedings of the Symposium held at Ridgefield, Connecticut, November, 1953)*. Academic Press, New York.

Data for Biochemical Research

Edited by R. M. C. Dawson, Daphne C. Elliott, W. H. Elliott & K. M. Jones. Oxford: Clarendon Press, 1959. xiii + 299 pages. 24 × 16 cm. £3 3s.

In their preface, the editors state that their aim has been to bring together as much information as possible about the compounds and reagents used by biochemists in their everyday research work in the laboratory.

The first 177 pages consist of a table of the properties of biochemical compounds. This table is divided into 19 parts covering such subjects as phosphate esters including nucleotides, carbohydrates and related compounds, water-soluble vitamins, enzyme inhibitors, metal-chelating agents and antimetabolites. There is also a section on miscellaneous compounds which contains information about such substances as ammediol, ninhydrin, sodium borate and benzyloxycarbonyl chloride. The more modern synonym for this last compound, benzylchloroformate, has, however, been omitted.

The subdivision of this table is unfortunate; one is frequently in a quandary as to which section to consult for a particular compound. Extensive use must therefore be made of the index, which is excellent. Would it not have been better and far more convenient, to arrange the material as a series of small paragraphs as in, for instance, *The Merck index of chemicals and drugs* (Merck, 6th ed., 1952)?

Apart from the usual properties such as molecular weight, solubility, and melting-point, methods of preparation and of estimation of many compounds, as well as formulae of all of them, are given. There are also extensive spectroscopic and pK_a data, particularly for the purines, pyrimidines, nucleosides and nucleotides. It is a pity that some of the pK_a values for cysteine, glutathione and tyrosine are erroneously assigned to various groups in the molecules.

The second section describes 71 enzymes which are commonly used as laboratory "reagents". Details are given of the reactions catalysed, of substrates attacked, of activators and prosthetic groups, and of principal inhibitors, as well as references to the preparation and use of these enzymes in assays.

Section III deals with buffers and physiological media. Data are given of standard pH solutions, the values, in general, being confined to the temperature range 25–38° C. It is a pity that no values of standards are given near 0° C., since one frequently has occasion to measure the pH of cold solutions. Details for the preparation of 27 buffers are given, these including several which have not previously been published. It is strange that the Clarke and Lubs buffers are taken from the original description in 1916 and that the more recent values for these same buffers, determined at the National Bureau of Standards, Washington, are not mentioned. This most useful section is completed with directions for the preparation of various mammalian and frog Ringer's solutions.

There follow 63 pages devoted to methods for the detection of biochemical compounds on paper. It is not clear what purpose this serves, since no details of chromatographic techniques are given and the information might as well have been included in the first table of properties of substances.

A number of short tables follow, giving data of ion-exchange resins, isotopes, manometry, and ammonium sulphate solutions, and there is a fascinating table of miscellaneous reagents, tests and recipes, acid-base indicators, normalities of concentrated solutions of acids and ammonium hydroxide, autoclaves, miscellaneous formulae and, at the end, atomic weights.

It is easy to criticize such a compilation because every practising biochemist will have his own ideas as to what should have been included and on how it should have been presented. Nevertheless this book contains a wealth of valuable information, in a handy and compact form, and will no doubt find its way into most biochemical laboratories, where it will be used extensively. One cannot, however, be anything but surprised that the index reveals no information in the book on carbon dioxide or on oxygen, both of which are so deeply involved in the life processes of man and many other organisms.

S. P. Datta

Achievement: Some Contributions of Animal Experiment to the Conquest of Disease

Geoffrey Lapage. Cambridge: W. Heffer & Sons Ltd., 1960. viii + 255 pages. 22 × 14 cm. 18s.

This book, written presumably for the non-scientist, tells "... what happens when scientists do experiments on living animals, what kinds of animals are used for these experiments and some, at any rate, of the benefits to man and animals that these experiments have conferred". After a brief description of scientific methodology, the author outlines the principles of immunity. Three chapters are given to diseases caused by bacteria, rickettsias, viruses, spirochaetes and parasitic animals, in which are described their symptoms, the ways in which they are transmitted and the methods by which they are attacked. Since man is ultimately the experimental animal in diseases to which he is subject, another chapter shows how he has been used in the investigation of such diseases, and also for studies on the effects of *x* rays, anaesthetics, hormones and abnormal changes in his environment. Similarly, domestic animals are used experimentally in the study of diseases which afflict them. Reference is made to the use of laboratory animals by the surgeon to test sutures and to develop techniques of grafting arteries, veins and bones; and by other workers to study such diverse problems as blood disorders, deficiency diseases, tumours, the action of hormones, and diseases affecting the ear and eye. Laboratory animals are also used for the standardization of toxins, antitoxins, immunological sera, some hormones and vitamins, and for the determination of the toxic properties of substances used in agriculture and industry.

The author has crammed many facts on this wide subject into 210 pages, and in parts the descriptions become little more than a catalogue. Since the story of the conquest of infectious diseases has been told before, one might have preferred to read more details on fewer subjects, showing how the scientists, using the principles outlined in the first chapter, formed their hypotheses, tested them by experiments, and arrived at their conclusions. This, while it would not have resulted in the author's "aeroplane's-eye view", might have made the book more useful to the student.

There are two important appendices, one outlining the regulations under the Cruelty to Animals Act (1876), amended in 1912, by which experiments on animals are governed. The second appendix, on the breeding and care of laboratory animals, describes the fine work of the Laboratory Animals Centre in ensuring that high standards are maintained in Great Britain for the production of animals for experimental purposes. This, together with the use of modern statistical techniques, ensures that as small a number of animals as possible is used to obtain the desired information. Although the author does not specifically justify experimental work on living creatures, it is plain that without such work the fight against disease would have been less successful.

There is an excellent bibliography for those wishing to read further.

A few errors occur. The suggestion that insulin, like thyroxine, cortisone and adrenaline, can be manufactured is incorrect; it is extracted from pancreas. On page 58 part of a sentence has been omitted. "Domagh", page 144, is usually written "Domagk".

G. A. Stewart

Encyclopaedia of Microscopic Stains

Edward Gurr. London: Leonard Hill (Books) Limited, 1960. xii + 498 pages; formulae. 25 × 16 cm. £4 15s.

Up to comparatively recent times microtechnical methods were based more on myth and lore than on established scientific facts. There has been also, especially in Great Britain, an almost complete divorce between the technicians who employed these methods and the research workers who made use of the information obtained with these empirical procedures.

Apáthy, who laid the foundations of the science of microtechnics, and in whose institute the present reviewer received his basic microtechnical training, was the first to realize the drawbacks of this schism. Though his pioneer work was not fully appreciated at the time, today research workers everywhere are actively engaged in assessing and evaluating the virtues and disadvantages of the older methods, and by the study of the underlying physico-chemical principles are developing newer ones.

One difficulty still remains. There is not enough liaison between microtechnicians and the makers of microscopic dyes. Many of the first group, quite understandably, are not sufficiently trained in the chemistry of dyes to be able to make logical use of the available stains, while most of the manufacturers are unable to appreciate the real needs of their customers.

Variations in the staining capacity of dyes produced by different makers, or changes in the tinctorial qualities of different batches of

the same dye produced by the same maker, present a permanent problem. Sometimes one suspects the presence of impurities in a certain dye, more often harmful than useful. At other times one feels that dyes masquerading under the same name are not identical in their chemical composition, but owing to the lack of reliable tests it has been impossible to remedy the situation.

It is largely owing to these facts that Mr E. Gurr's *Encyclopaedia of microscopic stains* is so welcome. He has, so to speak, a foot in both camps. For, as shown by the success of his previous two books, he is an experienced microtechnician and histochemist, who is fully aware of the needs and requirements of these disciplines, but at the same time has the additional advantage that he makes his own dyes and thus has the whole process from beginning to end under his control.

In his present work the reader will find not only the exact chemical structure of a dye molecule, but also details concerning its molecular weight and solubility in various media. This is the type of information every microtechnician is looking for—so far mostly in vain. Admirable though this is, further details concerning some of the other physical properties of the dyes would have been equally useful, and information derived from the chromatographic analysis of the various dyes should also have been included, but probably at the present stage of our knowledge all this would have unduly delayed the appearance of this long-awaited book.

Finally the author has brought together in a compact form much useful information concerning the histological and other uses of the various dyes, information which is mostly scattered in books and periodicals often not readily accessible. Apart from its other virtues this additional feature alone makes the work invaluable for every practising microtechnician.

P. Bacsich

Biochemistry in Relation to Medicine

C. W. Carter, R. V. Coxon, D. S. Parsons & R. H. S. Thompson. 3rd ed. London: Longmans, Green & Co., 1959. xiii + 628 pages; plates; figures. 22 × 14 cm. £2 10s.

A remarkable feature of the English and American languages is that, having common roots, they are so different. Nothing illustrates the matter better than this the third edition of *Biochemistry in relation to medicine*, an expanded and up-to-date version of its two predecessors.

I have compared it with three well-known American text-books of about the same range and aimed at the same audience. *Biochemistry in relation to medicine* says much the same as two, and more than the third, in two-thirds of the space. As a natural consequence it is something like half the cost. Why this should be is a mystery requiring a detailed analysis of the use of words that I have not the time or space to develop here. For, though this book is full of information, it is not crammed with it. Indeed it could be reduced by yet another 50 pages without materially reducing its value.

Two new authors have joined Drs Thompson and Carter since the last edition, to add specific chapters on the adrenal, on the thyroid and on reproduction, with a more general chapter on the part played by the endocrines in metabolic control.

The chapters on the body-fluids and renal function have been expanded and very much improved. All this has meant pruning of the old text in order to keep the book of reasonable size; and the authors have wisely, though no doubt with regret, remembering their comments in the first edition, struck out all the material dealing with classwork and experimental procedures that were included in the second edition.

It is hoped that in the next edition the authors will find time to make a thorough revision of the chapter on physical principles and methods. If they are to devote ten pages or more to the hydrogen ion and titration curves, would it not have been useful to include at this point in the book the meaning of equivalence in relation to valence, and to introduce here the concept of milli-equivalents that still appears to puzzle some older students and consultants?

Three pages are devoted to a sketchy outline of the fundamentals of thermodynamics, but nothing is said of the modern concepts of the chemical bond. These concepts are now fundamental to biochemistry, as they are to chemistry. Finally, if a beautiful plate is devoted to the appearance of the osazones of the simpler sugars, surely somewhere in the book space could be found for a brief outline of chromatography, a technique which has done so much to illuminate the biochemistry of the carbohydrates and proteins.

At the back of the volume, there are 50 pages of bibliography. It would be a great help to students if this were subdivided into original and review articles. It might, at the same time, be brought up to date. For example, there appears to be no reference to an excellent and thoughtful review on pH by Dr A. G. Ogston, published more than five years ago.

These are trivial criticisms, having regard to the general excellence of the book. Every technician in my Department of Chemical Pathology is expected to have read this book before proceeding to his final examinations; every postgraduate worker is expected to know the meat of the book; and we would hope that anyone aspiring to make a biochemical contribution to medicine today would regard the substance of this book as minimal reading. *Biochemistry in relation to medicine* is sound rather than stimulating. But perhaps today some teachers bow down too much to "stimulation" and, as a consequence, fail to give their students a "sound" base from which to expand their biochemical knowledge.

N. H. Martin

Clinical Physiology

Edited by *E. J. Moran Campbell & C. J. Dickinson.* Foreword by *Robert Platt.* Oxford: Blackwell Scientific Publications, 1960. xi + 530 pages; 29 figures. 23 × 15 cm. £2 10s.

In this book a group of clinicians has collaborated in writing on various aspects of clinical medicine to which physiology, as distinct from biochemistry, has contributed. The subjects dealt with include respiration, circulation, body-fluids, kidneys, skeletal muscle, the liver, the alimentary tract and the endocrine glands. The nervous system was not considered by the editors to be suitable for discussion at the level required by the general medical reader. Other subjects that might have been included, such as the skin, were doubtless omitted on the ground that some branches of physiology have not yet had any appreciable influence on the practice of medicine. In most of the chapters the author begins with a discussion of the physiology of the organ or system, in health and disease, and then describes very briefly the tests used for assessing function, with the main emphasis on underlying principle rather than practical detail.

The style of writing is of a high standard throughout, all the authors saying what they have to say in clear and unaffected English. The figures are clear, easy to understand from the legends, and not obscured by too much detail. The references at the end of each chapter consist of suggestions to the student for further reading, with what might be thought in some cases to be undue bias in favour of the author's own writings. A list of headings and subheadings at the beginning of each chapter would have helped the reader to keep his bearings more easily. The book has an index.

The title and contents of the book raise the question whether "clinical physiology" expresses a distinct point of view from which it was worth while writing another text-book. In the present state of knowledge it still seems possible to discuss respiration, the kidney, the circulation and the "mechanics" of the alimentary tract more or less comprehensively without going beyond the bounds of what is generally understood by physiology; to this extent some of the authors have written well-balanced chapters while keeping within the terms of reference of the book's title. In other cases, however, this is not so obvious. Though there are some aspects of the thyroid, bone-marrow and liver that could be called physiological, it would not have been reasonable to discuss them in isolation in a book of this sort. This may be why some of the authors have tried to cover rather too much ground in the limited space available to them. In the chapter on blood and bone-marrow, for instance, the author has surveyed practically the whole of haematology, with the exception of that part concerned with treatment. The result is a chapter that will be useful as an aide-mémoire for the medical student facing his final examination or the postgraduate taking a refresher course but is too condensed to serve as an introduction to the subject and not detailed enough for the specialist. Elsewhere in the book, failure to keep within the bounds of physiology has led to a certain sketchiness and lack of balance. It may be doubted, for instance, whether anyone but an expert should attempt to deal with the whole of carbohydrate metabolism in man in one page of a chapter on the liver. The success of this book will depend largely on how far it is found to satisfy a need not already filled by a book such as Best & Taylor's *The physiological basis of medical practice*, together with the special medical text-books already available, many of which are written by authors who have a good grasp of physiology and biochemistry.

N. B. Myant

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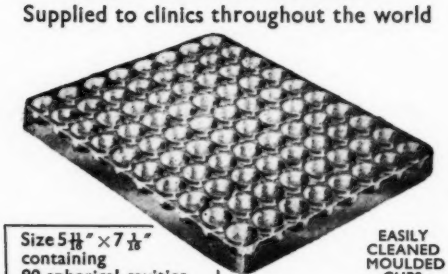

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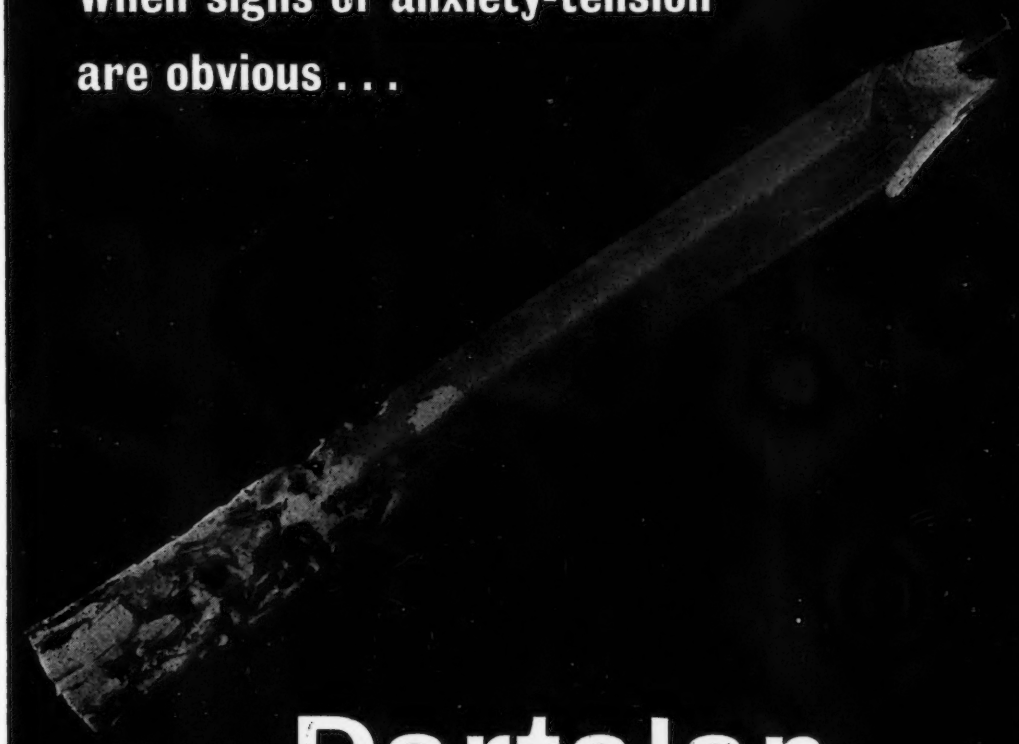

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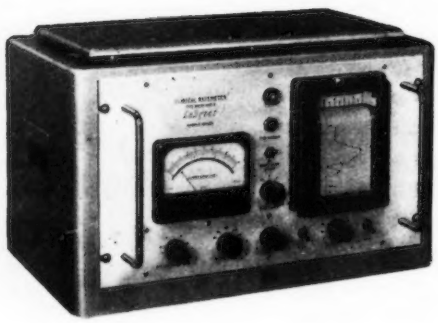

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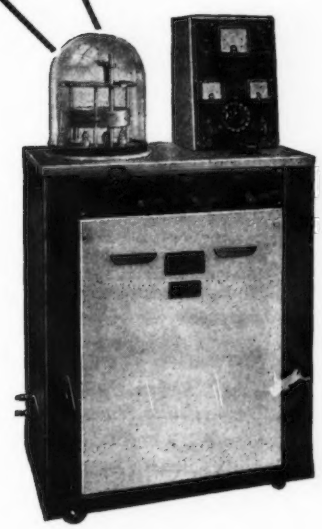

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References
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Ibid. 1016.
Lancet, 1958, **2**, 1084.
Arch. Int. Med., 1958, **102**, 998.
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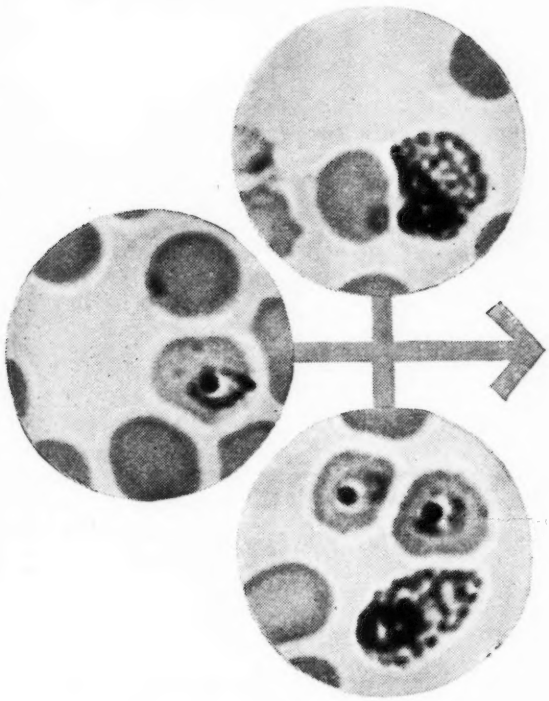

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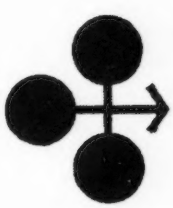

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